EVALUATING NOVEL THERAPEUTIC INTERVENTIONS FOR OSTEOARTHRITIS IN THE MURINE KNEE JOINT FOLLOWING ANTERIOR CRUCIATE LIGAMENT RUPTURE

by

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Title: Evaluating Novel Therapeutic Interventions after Pathological Degeneration of the Murine Knee Joint Following Anterior Cruciate Ligament Rupture

Approved: *Angela Lin, MSME*
Primary Thesis Advisor

Osteoarthritis (OA) is a degenerative joint disease that affects millions of US adults and costs billions of dollars to treat. OA causes joint immobility, pain, stiffness and, most importantly, has no cure. My primary research focus is a subdivision of OA, known as post-traumatic osteoarthritis (PTOA) that arises from joint trauma, like an anterior cruciate ligament rupture. It is essential to study ACL ruptures in small animal models, particularly rats, and use that as an approach to better understand the human disease process. The purpose of this study was to research how different types of therapeutics administered into the knee joint of a rat after an ACL rupture modify disease pathogenesis following the injury. To accomplish this, we used a non-invasive knee injury (NIKI) machine to create consistent ACL ruptures without direct damage to other tissues in the area. The NIKI allowed us to observe changes to cartilage and bone as early as 4 weeks post ACL rupture and presents biomarkers such as inflammatory cytokines. We then screened multiple promising therapeutic agents in vivo and longitudinally monitored anatomical/functional changes. We also explored the molecular underpinnings of these therapeutics using in vitro experiments. To do this, we exposed healthy cells from the knee joint synovium to different inflammatory agents (specifically Tumor Necrosis Factor and

Interleukins) and therapeutic drugs to produce an OA-like microenvironment. Expected outcomes of OA include cartilage loss, growth of bony spurs called osteophytes, and inflammation, and with the introduction of varying therapeutics we hope to see these degenerative effects be either prevented or decreased.

Acknowledgements

First and foremost, I would like to offer my thanks to the Guldberg lab for not only assisting me through my thesis process, but for also assisting me learn and grow as an undergraduate research assistant over the last few years. Without this lab's support and guidance, my thesis wouldn't have been possible.

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To Trond Jacobsen, I appreciate your commitment to me and openness to join my thesis committee through the Clark Honors College. While our academic areas of expertise differ a little, there is no one else in the Honors College I would want in my corner throughout this process.

To my friends and family, I honestly don't think I could have completed my undergraduate career without both of your support. Thank you for being there through the ups and downs of my career, it has truly given me belief in my future and obtaining my goals.

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Introduction:

Osteoarthritis (OA) is a degenerative joint disease that is categorized as the most common form of arthritis; the primary occurrence of OA being when the protective sheath of cartilage that covers the ends of bones in a joint breakdown (*Osteoarthritis*, n.d.). OA affects over 27 million people in the US alone and costs approximately $185.5 billion to treat (*Insurer and Out-of-pocket Costs of Osteoarthritis in the US*, n.d.), and there is no cure. For those who have to suffer from the burden of OA, their quality of life is significantly reduced as characterized by pain and limited activity. Because of these hindrances, there is a clinical need to develop and test novel OA interventions that can modify the underlying disease pathology to pathologically cure or delay the disease, allowing individuals to better live their lives.

With OA, the two most important risk factors for developing this disease consist of obesity and joint injury (Hunter et al., 2020). With joint injury being a prominent risk factor in developing OA, it is extremely relevant to research OA that develops after injury to the joint, otherwise known as Post-traumatic Osteoarthritis (PTOA). The injury of a joint is extremely prevalent in modern day sports in athletes, and it is known that athletes are at increased risk to develop PTOA compared to more sedentary populations (Carbone & Rodeo, 2017). In fact, those who develop PTOA are generally younger, healthier, and more active than those who develop idiopathic OA (Carbone & Rodeo, 2017); the most common athletic injury that puts patients at the highest risk of developing PTOA being an anterior cruciate ligament (ACL) rupture, where roughly 50% of ACL injured knees progress to OA within 5-15 years (Friel & Chu, 2013). Since there is currently no cure to OA or PTOA, it is essential to research how to delay the progression of OA once an individual begins to suffer from it or potentially prevent it from occurring to begin with. A wide array of works have sought to address this in recent years via a number of

regenerative and rehabilitative approaches. Common approaches to attempt to further understand the relationship between OA and its unfortunate side effects consist of conducting experiments in rodent models in an attempt to not only hope to correlate findings to that of humans, but to also provide possible evidence for if, when, and how a molecule, cell, cytokine, or drug is prevalent in OA progression or hinderance (Zaki et al., 2022). We have preliminarily identified candidate novel therapeutics based on literature, and our goals are to study their effects in an *in vitro* microenvironment and *in vivo* in a rodent model of ACL rupture (ACLr) on OA-relevant tissues. In vitro analysis and screening were conducted to investigate potential mechanisms of therapeutic action and explore effects at a cellular level.

To explore the relationship between candidate drugs and PTOA-relevant tissue changes, and to determine if these same drugs produce a therapeutic effect, one route is to establish a workflow in an *in vitro* model, outside of a living organism, and then progress to an *in vivo* model, within a living organism. *In vitro*, we are able to screen therapeutics to measure varying important PTOA biomarkers, and *in vivo* we are able to incorporate a preclinical animal model to test more pathological changes in the knee. A primary indicator of OA pathology is the presence of chronic inflammation, which is a driver for pain, joint immobility, and cartilage degeneration (Daghestani & Kraus, 2015).

Our group has worked with a variety of therapeutic approaches aimed at regulating inflammation, the immune response, and tissue regeneration. For example, the delivery of the molecule Resolvin D1 (RvD1), which is a specialized pro-resolving mediator that helps resolve inflammation and promote healing after an injury or infection (Li et al., 2020), post ACLr can be used to attempt to control inflammation and the immune response. Platelet Derived Growth Factor (PDGF) is another novel therapeutic that has prominence in a variety of orthopedic

applications due to its capacity for tissue repair (Cai et al., 2023), and has been found to have the potential to protect cartilage in the context of OA and reduce inflammation. Our final therapeutic of interest is Ferulic Acid (FA), which has been found to have a significant role in preventing IL-1β (a pro inflammatory signaling molecule in OA pathogenesis) induced OA chondrocyte toxicity (Du et al., 2021). In order to administer these drugs into the synovial joint of the knee, we administered RvD1 and PDGF in either a free drug or microgel encapsulated form; where a free drug delivery should result in a quicker, less targeted delivery, and the microgel form resulting in a slower, more targeted delivery. The comparative baseline of this study was labeled Sham, essentially the "Placebo" group where rats did not undergo ACLr and did not receive any of the previously mentioned therapeutics. *In vitro* testing consisted of testing the interaction of various drugs with synoviocytes, cells that line synovial joints (in this specific scenario knees), and measuring varying types of inflammatory markers such as the presence of Nitric oxide and nitric oxide synthase, changes in metabolic activity, and DNA content. Before running tests *in vivo*, it is important to have a basic understanding of these potential interactions *in vitro*. It is crucial to have a specific idea of the effects drugs/cytokines will have on a targeted joint's tissue; quantifying drugs and cytokines within the synovial membrane can be studied via 2D *in vitro* cell culture methods. *In vivo*, it is essential to have an animal model of OA that represents human OA manifestations in order to test therapeutic efficacy.

Many *in vivo* PTOA studies utilize the incorporation of a surgical model to create an injury to the joint. While this technique proves effective, it can result in unwanted variables during recovery such as an increased immune response and increased inflammation. Our particular studies utilize a non-invasive knee injury (NIKI) device to better represent the human disease induction inflammatory environment. The NIKI removes complicating factors that

accompany surgery and better prevent these confounding variables. In this model, an axial force is applied distally to the femur superior to the knee joint that ruptures the ACL, ultimately causing joint instability and PTOA pathology. The NIKI ensures an ACLr without direct damage to other tissues.

Our goal was to attempt to observe how the incorporation of novel therapeutic drugs *in vivo* and *in vitro* effect OA pathology with the primary focus of identifying if these drugs demonstrate therapeutic efficacy with possible potential to improve quality of life. *In vitro*, we planned to compare the functional therapeutic effect of RvD1 and PDGF on synoviocyte cells and the various inflammatory biomarkers that arise from it. *In vivo*, we planned to observe PTOA pathological changes in the knees of rats upon ACLr and further assess if incorporation of the previously mentioned therapeutics displayed therapeutic efficacy within a preclinical animal model. Ultimately, it is important to incorporate the previously stated long term goals to attempt to improve the socioeconomic impact that OA and PTOA has on individuals who are burdened with this disease. Similarly, it is important to lessen the burden on the struggling health-care system and make fundamental changes to the way we care for individuals with OA (Hunter et al., 2014).

Methods:

In-vitro Methodology:

The Guldberg Lab uses HIG82 Synoviocyte cells, cells that are found within the synovial membranes of rabbits' synovial joints. HIG82 synoviocytes have been found to have important interactions with cytokines we plan on testing (IL1 and TNF-α) (Georgescu et al., 1988), thus making it an acceptable cell line and model for studying both how OA progresses at a cellular

level and as a way to screen therapeutics. Both IL1 (Interleukin 1) and TNF-α (Tumor Necrosis Factor) are proinflammatory cytokines that are naturally released as an immune response (Dayer, 2002).

Synovial inflammation is a common side effect of OA, PTOA, and arthritis in general (Goldhammer et al., 2010), and can be used as a model system to run experiments on. In this model, cells were passaged prior to 80% confluency to prevent phenotypic changes that happen with over proliferation and to ensure enough nutrients is being administered to said cells; we use an F-12 nutrient mixture containing L-glutamine combined with penicillin-streptomycin (Pen-Strep) and fetal bovine serum (FBS). Pen Strep is used to incorporate an antibiotic solution that is helpful with cell culture and treating infections in animals (Martínez-Liarte et al., 1995), and FBS is used to support cell growth and maintenance (*Fetal Bovine Serum - an Overview | ScienceDirect Topics*, n.d.). The combination of these three are known as "cell media".

Synoviocyte cells were plated within an 75cm2 flask with a seeding density of 10,000 cells/cm2 and at a confluency of around 70-80%, meaning that when roughly 70-80% of the surface area of the culture dish is covered by cells, they are transferred to a new dish to continue proliferation (Segeritz & Vallier, 2017). This process was repeated bi-weekly to prevent over-proliferation and to set up potential experiments by plating synoviocytes within a 96 or 48 well plate. Plating synoviocytes includes the same previous seeding density of 10,000 cells/cm2, however the area per well is different than the 75cm2 flask. 96 well plates have a 0.32cm2/well area and the 48 well plates have a 1.1cm2/well area; calculations were made accordingly to get the desired number of cells within each plate:

(Seeding Density)(Area/Well)(#Wells)=cells needed

48 well plate: (10,000cells/cm2)(0.32cm2/well)(32wells)=102400 cells total

While passaging the synoviocytes, counting the number of cells is crucial in order to finish plating in a well plate. This was done by centrifuging the cells to separate the synoviocyte cells from the cell media, resuspending the newly formed cell pellet within one mL of media, and getting a cell count of how many cells we have per mL. With this calculation, the final calculation can be done to finish plating.

(102,400cells)(5.27x106cells/mL)=.0194mL (5.27E6 being an example cell count from a previous study).

After plating the synoviocytes, we treated each well with different concentrations of drugs/cytokines of interest to measure different types of content assays. For this therapeutic study, we implemented the usage of RvD1 and Iloprost (another anti-inflammatory drug (Müller et al., 2010)) as our main drugs, and IL1 and TNF-α as our cytokines. Plating the cells occurs on the same day as passaging, 24 hours later pretreating occurs with drug of interest, followed by another 24 hour incubation period before treating with cytokines. After this process, different assay tests were performed to measure varying outcomes of interest, such as DNA, Nitrite, Nitric Oxide Synthase (NOS), and metabolic activity content. Each of the previous were measured using different kits to extrapolate data appropriately.

At the end of each experiment, the cell culture media previously used can be utilized to perform such assay tests as listed prior. The cell media was saved in either a -20℃ or -80℃ storage freezer or used immediately to run tests in a separate well plate from the adhered cells from previous plating. Each assay test has varying guidelines in regard to how to run each one primarily because they each measure different variables. The cells that were adhered in the original plate were either fixed in formalin (a diluted form of formaldehyde) to maintain their structure and morphology for further analysis (*Preparing Fixed Cells for Labeling - US*, n.d.) or

frozen alongside the media. When cells become frozen, they are permeable and preserved in a viable state; this can be used as a cell bank for future research needs. It is important to practice proper cryopreservation techniques, as the cells will likely be damaged or killed due to the formation of ice crystals inside them, which will ultimately disrupt cellular structures and membranes, and leading to cell death when thawed (*Cryopreservation Basics*, n.d.).

Assay Experimentation:

DNA: The "CyQuant Cell Proliferation Assay" is an important test to measure the amount of total DNA content for a certain number of cells. More importantly, it can be used to provide an accurate and simple measure of cell number, as DNA content is directly proportional to the amount of cells (i.e, more DNA content means more cells). The results for the DNA Cell Proliferation Assay were compared against a known standard curve.

Nitrite Assay: The Nitrite Assay kit is used for accurate quantification of nitrite by measuring NOS content, which is a very important marker for inflammation. One of the primary factors of inflammation is the presence of NOS, which is an enzyme that catalyzes the production of NO from L-arginine, a common amino acid found in our body. NO is highly reactive due to its unpaired electron, thus making it very inflammatory when in contact with anatomical features of the body. The measurement of NO is very complicated due to its instability, however in order to create NO, NOS must be present as well. The results for the Nitrite Assay kit were compared against a known standard curve.

Metabolic Activity: The Alamar Blue assay is used for various measurements, but the primary ones being cytotoxicity, cell proliferation and cellular metabolic activity. Alamar blue assay and DNA content measurements go hand in hand, as a higher value (usually measured in arbitrary units) indicates more cells. When Alamar blue is added, it is metabolized by the cells it comes in

contact with, ultimately changing the color of the dye. When there is more metabolic activity or more total cells, there is more of a difference in the color of the dye and can be measured via fluorescent plate reader.

In order for the previously stated variables to be measured, the incorporation of fluorescence spectroscopy is needed. In cell cultures, fluorescence light works by "illuminating" a sample with a specific wavelength of light that excites molecules within the cells. These excited cells emit a wavelength of their own that allows researchers to identify specific cellular components by selectively labeling them with a fluorescent dye or protein. The Guldberg lab uses a fluorescent plate reader to measure these fluorescence levels and give us a basic understanding of the quantity of desired products.

NIKI Device:

Skeletally mature Male Lewis rats (13 weeks, 330g +/- 21g) were used for this particular study. In order to induce a non-invasive ACL rupture of these rats, a non-invasive knee injury (NIKI) device was used. The NIKI holds the tibia of each rat perpendicularly to the ground, with pressure applied to the knee using a custom cup, while maintaining a constant angle on the calcaneus within a foot/ankle holding slot. Rats were anesthetized with 2-3% isoflurane and injected with slow-release buprenorphine as an analgesic for 72 hrs. Upon confirmation of a steady grip of the rats leg, approximately 5N of force is applied to the distal end of the femur that increases by 5N every 10 seconds, pushing it down (Brown et al., 2020). ACLr was determined via translocation of the distal femur out of the knee cup; after ACLr, the rats were assessed for ACLr knee joint laxity and for potential bone fractures.

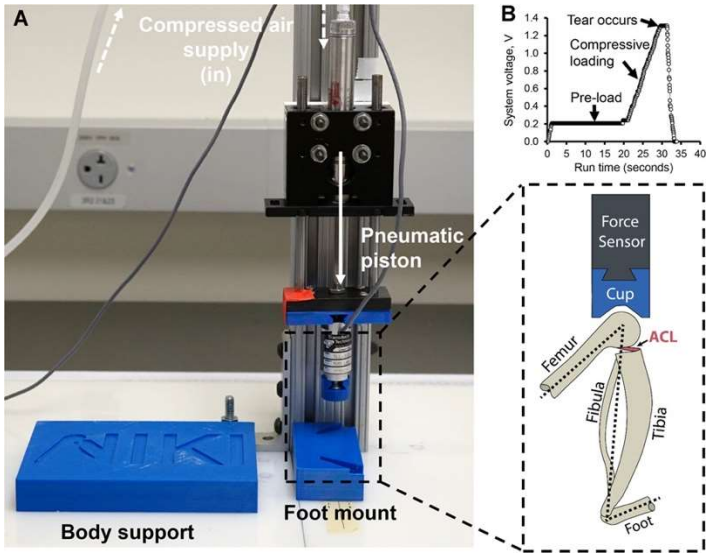

Figure 1: NIKI device (A), loading scheme including preload, compressive loading, and release of compression after rupture (B) (Brown et al., 2020).

Therapeutic Intervention:

One week post ACLr, varying types of therapeutic agents were administered into the intra articular space of each rat's knees. The following drugs were used: Platelet derived growth factor (PDGF) to help regulate cell proliferation and evoke injury response mechanisms (*Platelet-Derived Growth Factor - an Overview | ScienceDirect Topics*, n.d.), Resolvin D1 (RvD1) a lipid mediator with significant anti-inflammatory properties (Roohbakhsh et al., 2022), and Ferulic Acid (FA) another strong anti-inflammatory agent. RvD1 and PDGF were either administered in a "free drug" form or in encapsulated microgel particles. The incorporation of microgel drug administration was used to enhance a more targeted delivery mechanism to the injured area, despite that usage of microgel may not be as "strong" as free drug delivery. PDGF and RvD1 in both free and microgel form were administered at a concentration of 100ng/ml, whereas Ferulic Acid was administered at 15mg/ml. Therapeutic efficacy was measured longitudinally throughout the study by quantifying limb weight bearing functionality.

Dynamic Weight Bearing (DWB):

Weight bearing on each limb was determined at four different time intervals: one week, three week, and four week post therapeutic intervention and injury induction and one week prior to injury induction. In order to measure weight bearing, we used the BioSeb Dynamic Weight Bearing System 2.0. This system measures paw placement and weight using automated floor sensors with a camera to record real-time animal activity (Dent et al., 2023). This allowed us to obtain detailed information about how much weight is being born on each limb, injured or uninjured, front or hind, etc. Upon data collection, users go back into the BioSeb system manually to ensure enough time points were taken based on BioSeb's confidence grading scale.

µCT Analysis:

Post mortem, each leg was preserved in formalin to preserve tissues within the joints. Each knee was isolated and prepared for µCT analysis by carefully dissecting the femora, tibiae, and patellae away from each other while retaining the bulk of the synovium around the distal femur. Femurs, tibias, and patellas were all scanned in order to quantify cartilage values alongside subchondral bone (SCB) density. In order to scan these bones, it is important to soak them in a conray/PBS solution; Conray is an anionic contrast agent that is used for soft tissue enhancement in certain CT, X-ray, and fluoroscopy imaging procedures (*Conray® (Iothalamate Meglumine Injection USP 60%)*, n.d.), which equilibrates in an inverse relationship with proteoglycan content in cartilage. After allowing the bone of interest to soak for approximately 30 minutes, the sample was gently patted dry, placed in a 15ml conical tube, and surrounded with packing foam used to stabilize the bone in place during the scan. Upon µCT completion, scans were formatted in a transverse cut, of which were then reconstructed sagittally to better quantify bone and cartilage tissue pathology changes. After this initial reconstruction, we were

then able to generate a colorized, 3-D image of each tibia, femur, and/or patella and isolate any region of interest (i.e medial plateau cartilage of the tibia, where darker colors such as red signify thicker cartilage and blue/green signifies thinner cartilage). Focusing on different regions of the bone is important; the medial tibiofemoral compartment is known to bear around 60% of the knee's weight and is thus more important to observe pathological changes compared to the lateral plateau (Malik et al., 2024). The previously stated process is essential in obtaining a reasonable understanding of the pathological changes occurring in the knee joint and how therapeutics alter pathology post ACLr.

Results:

In Vitro Results:

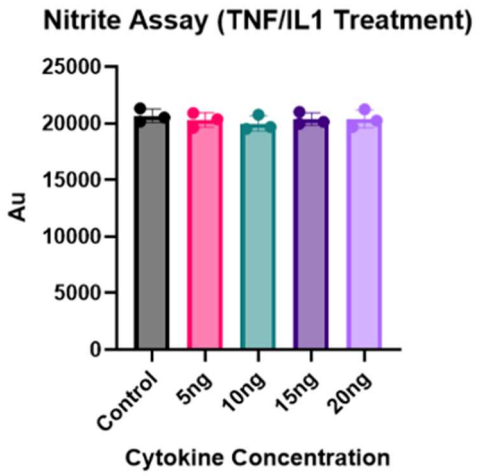

Figure 2: Testing of increasing cytokine concentration on presence of NO. An ANOVA was run, * denoting significance

Three different Nitrite assay trials were conducted for the *in vitro* aspect of this study. Each study was conducted with the addition of similar concentrations of inflammatory cytokines TNF-α and IL-1 in intervals of 5ng up to 20ng. Data was collected for the three separate experiments, averaged together, and plotted.

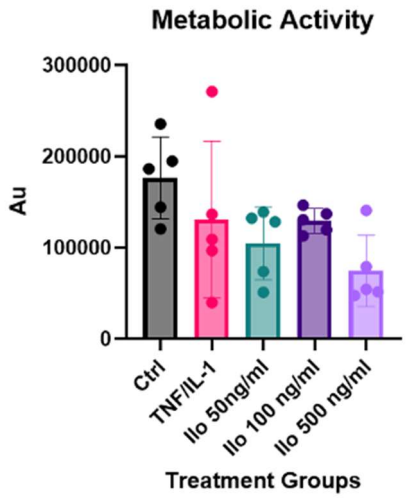

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Figure 3: Testing of increasing metabolic activity on presence of TNF/IL1, Iloprost. An ANOVA was run, * denoting significance

Only one Alamar blue assay was run to measure metabolic activity treated with various therapeutics and cytokines. Treatment groups consisted of TNF/IL1 in order to include similar variables to that of our Nitrite Assay, alongside varying concentrations of Iloprost. Metabolic Activity was measured in Arbitrary units by our fluorescent plate reader.

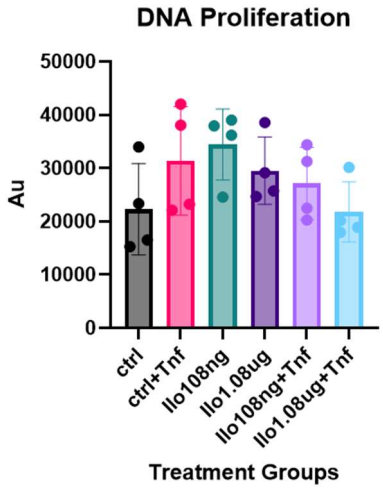


Figure 4: Testing of increasing cell proliferation in the presence of TNF/IL1, Iloprost. An ANOVA was run, * denoting significance

Only one Cyquant DNA assay was run to measure cell proliferation treated with various therapeutics and cytokines. Treatment groups consisted of TNF/IL1 alongside varying concentrations of Iloprost. Cell proliferation was measured in Arbitrary units by our fluorescent plate reader.

In Vivo Results:

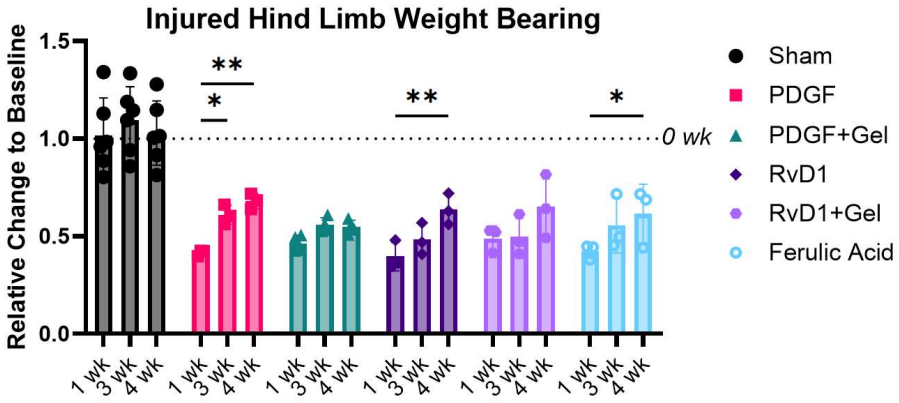


Figure 5: Weight bearing of animals on their injured, ACLr, limb 1 week, 3 weeks, and 4 weeks post injury. An Ordinary one-way ANOVA test was run, * denoting statistical significance (*$p<0.05$)

Data points are relative to the weight of each individual animal by plotting a ratio of animals' baseline, uninjured weight bearing data to their weight bearing at all other time points

3D reconstruction of tibias and femurs resulted in four primary results. Qualitative values of cartilage loss and growth in both the Medial Tibial Plateau and Medial Femoral Condyle were obtained and varying pathological changes showcased in Figures 3 and 4, alongside qualitative values of Subchondral bone (the layer of bone directly under the sheath of cartilage) in both the Medial Tibial Plateau and Medial Femoral Condyle in Figures 5 and 6. All of the following figures depict the utilization of five therapeutics previously mentioned in the Introduction and the visible changes in pathology that occurred.

Visualization of Cartilage Pathology:

Regarding the cartilage visualizations, a color coding scheme was utilized to highlight pathological changes and further showcase variations in cartilage thickness and distribution across different treatment groups. The darker region (signified by red) shows regions of the tibia with thicker cartilage tissue, whereas the lighter areas (green or yellow) indicate thinner cartilage

regions. All measurements were shown in mm with the bounds set at 0.00-0.250mm (0.00 being the lighter colors, and ≥0.250 being the darker colors). Data was obtained via sagittal reconstruction analysis.

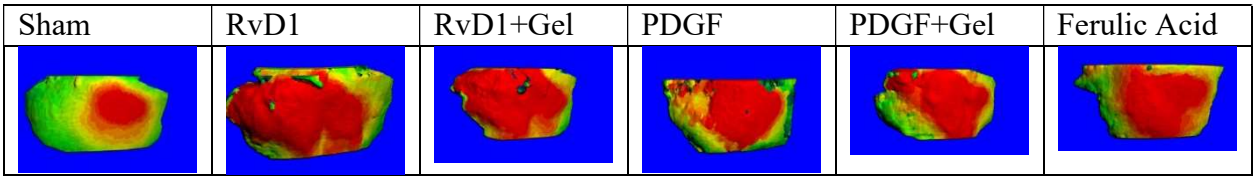


Figure 6: Cartilage growth and/or degeneration in the medial tibial plateau post ACLr upon therapeutic intervention.

The top of each image represents the lateral aspect of each tibia, and as the image progresses downward it is approaching the most medial area of the medial plateau. The leftmost area of each image is the posterior aspect of the tibia, and the rightmost is the anterior aspect.

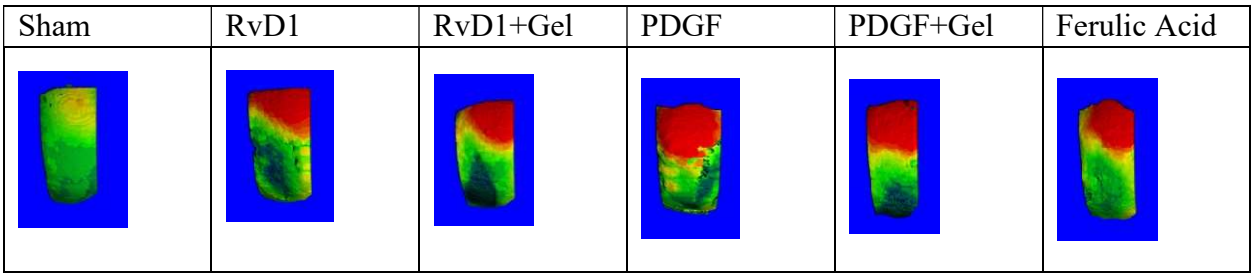


Figure 7: Cartilage degeneration and growth in the medial femoral condyle post ACLr upon therapeutic intervention.

The top of each image represents the more distal aspect of each condyle (closest to the knee joint), and the bottom of each image represents the more proximal aspect of each condyle. The rightmost aspect of each image represents the lateral feature of the femur, and progressing left nears the medial point.

Visualization of Subchondral Bone Pathology:

Changes in Subchondral bone pathology were formatted differently in regard to tibias and femurs. Both figures depict bone changes in the medial region of the tibial subchondral plate and femoral condyle subchondral plate, however the orientation of both vary. In the tibia, we obtained data via analysis of a coronal reconstruction of the tibia. With this view, it is easier to observe osteophyte formation and potential degeneration of bone in both cortical and trabecular

layers (both of which are seen as subchondral, as they both exist under a layer of cartilage). Subchondral analysis for femurs were run in similar sagittal processes as the cartilage evaluations.

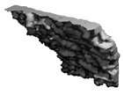
Sham	RvD1	RvD1+Gel	PDGF	PDGF+Gel	Ferulic Acid
					

Figure 8: Changes in trabecular and subchondral bone in the medial tibial plateau of rat knees following an anterior cruciate ligament (ACL) rupture.

The images compare bone architecture across different therapeutic interventions, highlighting structural variations in response to treatment. The top of each image represents the lateral aspect of each tibia, and as the image progresses downward it is approaching the most medial area of the medial plateau

Sham	RvD1	RvD1+Gel	PDGF	PDGF+Gel	Ferulic Acid
					

Figure 9: Subchondral bone changes in the medial condyle of the femur following ACLr.

The top of each image represents the more distal aspect of each condyle (closest to the knee joint), and the inferior aspect of each image represents the more proximal aspect of each condyle. The rightmost aspect of each image represents the lateral feature of the femur, and progressing left nears the medial point.

Cartilage Analysis:

With changes of cartilage in the tibial plateau, we prioritized highlighting changes in cartilage volume, cartilage attenuation, and cartilage thickness. Cartilage volume is measured as the total volume of cartilage within the measured compartment or sub-compartment. Cartilage attenuation is categorized as an estimate for cartilage quality, where a higher value signifies

poorer quality of cartilage due to decreased proteoglycan content. Finally, cartilage thickness refers to the relative distance between the top of the cartilage to the bottom of it at any given point, averaged across the region of interest. In the scope of previously run *in vivo* ACLr studies, both cartilage volume and thickness has been shown to typically increase in early stages of disease progression before it erodes away. Historical data of ACLr alone with no therapeutic administration was added to the graphs to provide an easier visualization of therapeutic efficacy. Statistical comparisons were made between these metrics using GraphPad, a graphical analysis software capable of creating charts, tables, and running statistical tests between groups. Statistically different groups are summarized in the tables below.

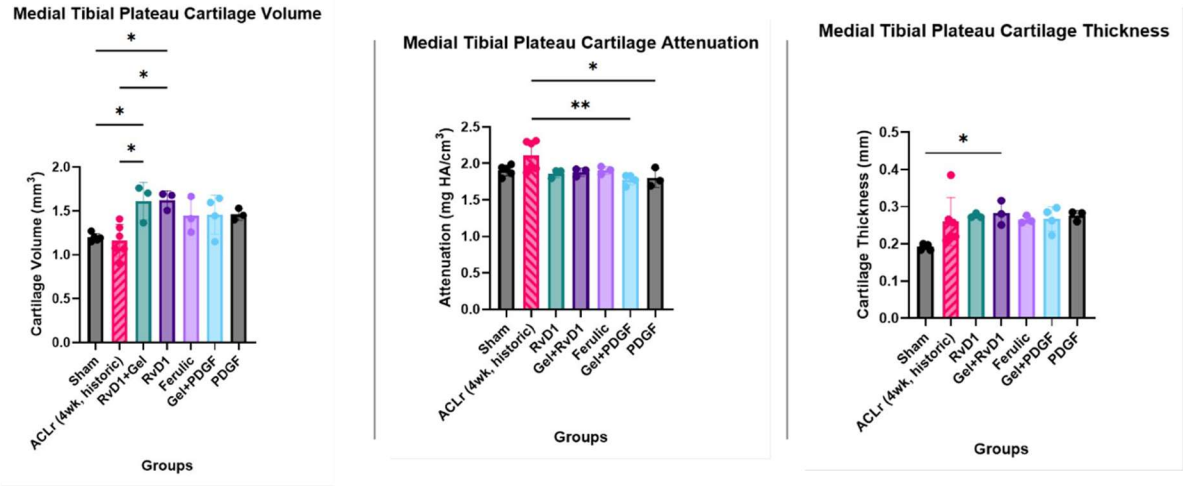


Figure 10: Testing of therapeutic efficacy with pathological changes to cartilage in Medial Tibial Plateau. (A) Cartilage Volume (B) Cartilage attenuation (C) Cartilage thickness. An Ordinary one-way ANOVA test was run, * denoting statistical significance (*p<0.05)

Medial Tibial Plateau Cartilage Volume

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Sham vs. RvD1+Gel	-0.4130	-0.7827 to -0.04320	Yes	*	0.0245
Sham vs. RvD1	-0.4282	-0.7980 to -0.05847	Yes	*	0.0190

Table 1: P-values for Medial Tibial Plateau Cartilage Volume

Medial Tibial Plateau Cartilage Thickness

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Sham vs. RvD1	-0.08332	-0.1318 to -0.03482	Yes	***	0.0006
Sham vs. Gel+RvD1	-0.09049	-0.1390 to -0.04199	Yes	***	0.0003
Sham vs. Ferulic	-0.07292	-0.1214 to -0.02442	Yes	**	0.0022
Sham vs. Gel+PDGF	-0.07492	-0.1195 to -0.03037	Yes	***	0.0008
Sham vs. PDGF	-0.08395	-0.1325 to -0.03546	Yes	***	0.0006

Table 2: P-values for Medial Tibial Plateau Cartilage Thickness

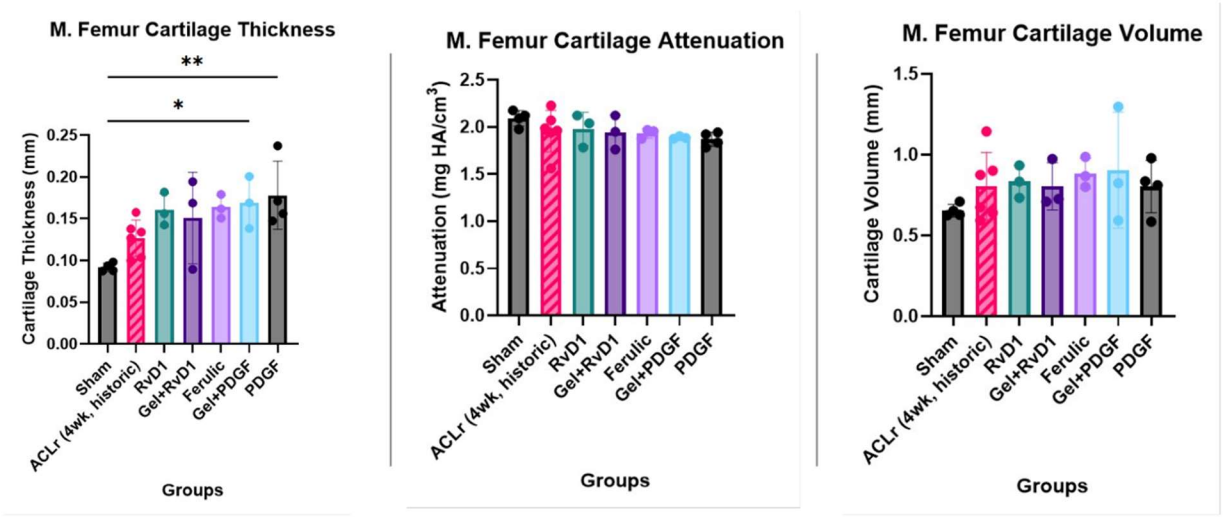


Figure 11: Testing of therapeutic efficacy with pathological changes to cartilage in the Medial Femoral Condyle. (A) Cartilage Volume (B) Cartilage attenuation (C) Cartilage thickness. An Ordinary one-way ANOVA test was run, * denoting significance (*p<0.05).

Medial Femoral Cartilage Thickness

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Sham vs. Gel+PDGF	-0.07745	-0.1516 to -0.003312	Yes	*	0.0371
Sham vs. PDGF	-0.08615	-0.1548 to -0.01751	Yes	**	0.0086

Table 3: P-values for Medial Femoral Condyle Cartilage Thickness

Subchondral Bone Analysis:

Shifting toward analysis of the subchondral bone, the four primary measurements utilized to objectify bone changes were changes in BV mean/density, BV direct, and Tb.th direct. BV mean/density represents the amount of mineralized bone in a given area, reflecting the bones overall density. A higher value of this measurement would indicate denser bone due to more mineralization. BV direct pertains to just the bone volume, how much space it is occupying. Finally, Tb.th refers to the thickness of the trabecular bone, of which is important to utilize in analyzing bone microarchitecture and potential fracture risk.

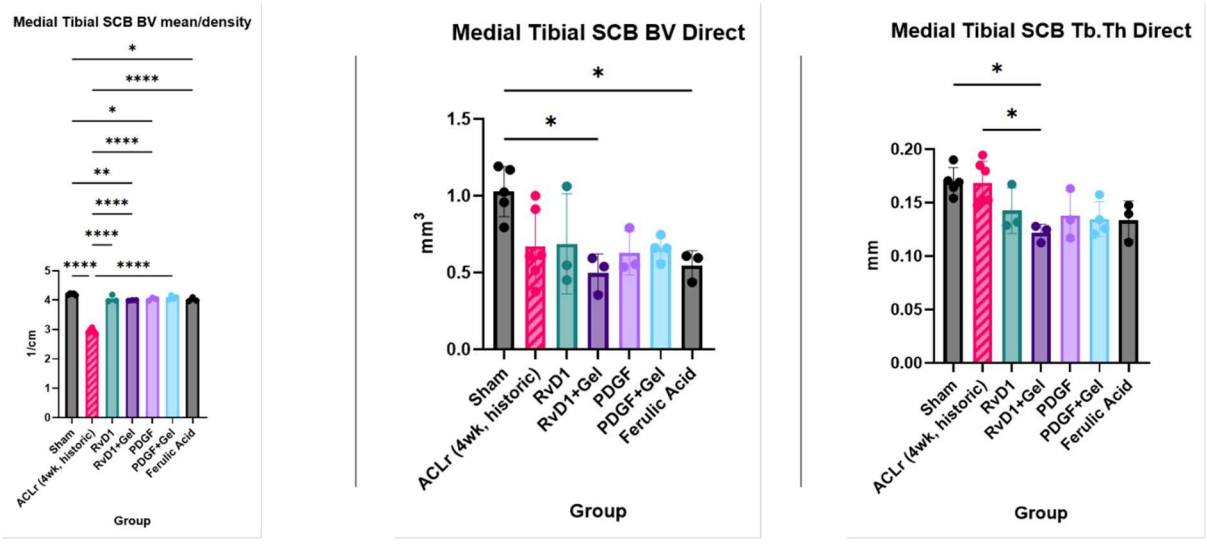


Figure 12: Testing of therapeutic efficacy with pathological changes to SCB in the Medial Tibial Plateau. (A) Bone volume mean/density (B) Direct bone volume (C) Trabecular thickness. An Ordinary one-way ANOVA test was run, * denoting significance (*p<0.05).

Medial Tibial SCB BV Direct

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
Sham vs. RvD1+Gel	0.5315	0.08035 to 0.9826	Yes	*	0.0146
Sham vs. Ferulic Acid	0.4805	0.02935 to 0.9316	Yes	*	0.0322

Table 4: P-values for SCB Medial Tibial Plateau BV direct

Medial Tibial SCB BV mean/density

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
Sham vs. ACLr (4wk, historic)	1.259	1.122 to 1.396	Yes	****	<0.0001
Sham vs. RvD1+Gel	0.2109	0.04558 to 0.3763	Yes	**	0.0073
Sham vs. PDGF	0.1700	0.004677 to 0.3354	Yes	*	0.0414
Sham vs. Ferulic Acid	0.1876	0.02221 to 0.3529	Yes	*	0.0199
ACLr (4wk, historic) vs. RvD1	-1.101	-1.261 to -0.9413	Yes	****	<0.0001
ACLr (4wk, historic) vs. RvD1+Gel	-1.048	-1.208 to -0.8882	Yes	****	<0.0001
ACLr (4wk, historic) vs. PDGF	-1.089	-1.249 to -0.9291	Yes	****	<0.0001
ACLr (4wk, historic) vs. PDGF+Gel	-1.137	-1.283 to -0.9905	Yes	****	<0.0001
ACLr (4wk, historic) vs. Ferulic Acid	-1.072	-1.232 to -0.9116	Yes	****	<0.0001

Table 5: P-values for SCB Medial Tibial Plateau BV mean/density

Medial Tibial SCB Trabecular Bone Thickness Direct

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Sham vs. RvD1+Gel	0.04789	0.005623 to 0.09016	Yes	*	0.0201
ACLr (4wk, historic) vs. RvD1+Gel	0.04700	0.006072 to 0.08793	Yes	*	0.0180

Table 6: P-values for SCB Medial Tibial Plateau Trabecular Thickness

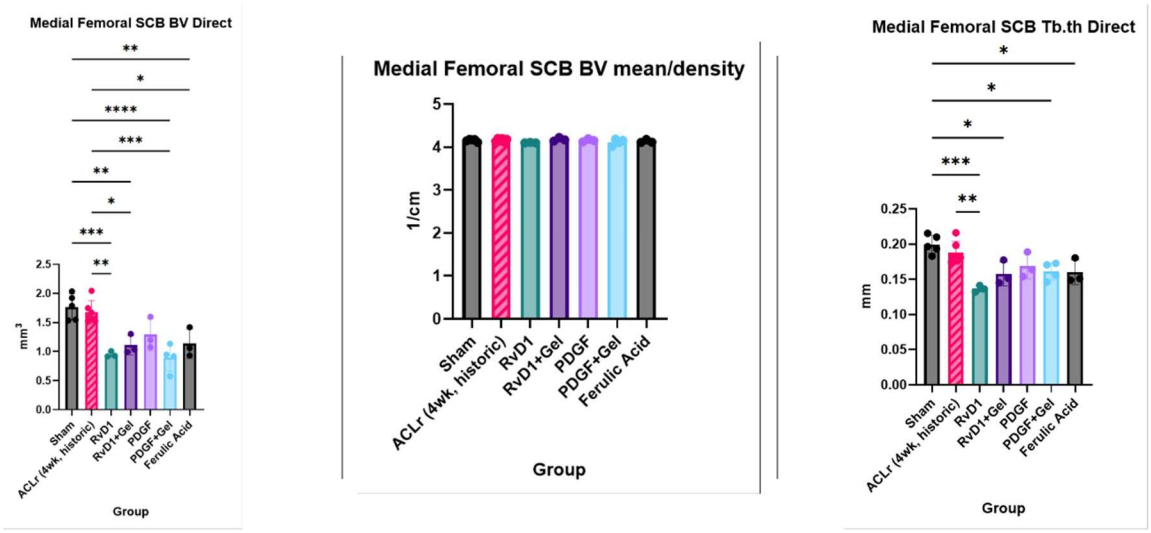


Figure 13: Testing of therapeutic efficacy with pathological changes to SCB in the Medial Tibial Plateau. (A) Bone volume direct (B) Bone volume mean/density (C) Trabecular Thickness. An Ordinary one-way ANOVA test was run, * denoting significance (*p<0.05).

Medial Femoral SCB Trabecular Thickness

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
Sham vs. RvD1	0.06313	0.02782 to 0.09844	Yes	***	0.0002
Sham vs. RvD1+Gel	0.04167	0.006358 to 0.07698	Yes	*	0.0144
Sham vs. PDGF+Gel	0.03823	0.005792 to 0.07066	Yes	*	0.0146
Sham vs. Ferulic Acid	0.03947	0.004158 to 0.07478	Yes	*	0.0223
ACLr (4wk, historic) vs. RvD1	0.05147	0.01728 to 0.08565	Yes	**	0.0014

Table 7: P-values for SCB Medial Femoral Condyle Trabecular Thickness

Medial Femoral SCB BV direct

TUKEY'S MULTIPLE COMPARISONS TEST	MEAN DIFF.	95.00% CI OF DIFF.	BELOW THRESHOLD?	SUMMARY	ADJUSTED P VALUE
Sham vs. RvD1	0.8153	0.3100 to 1.321	Yes	***	0.0006
Sham vs. RvD1+Gel	0.6553	0.1501 to 1.161	Yes	**	0.0062
Sham vs. PDGF+Gel	0.8706	0.4065 to 1.335	Yes	****	<0.0001
Sham vs. Ferulic Acid	0.6287	0.1235 to 1.134	Yes	**	0.0091
ACLr (4wk, historic) vs. RvD1	0.7280	0.2387 to 1.217	Yes	**	0.0016
ACLr (4wk, historic) vs. RvD1+Gel	0.5680	0.07882 to 1.057	Yes	*	0.0165
ACLr (4wk, historic) vs. PDGF+Gel	0.7833	0.3367 to 1.230	Yes	***	0.0002
ACLr (4wk, historic) vs. Ferulic Acid	0.5414	0.05218 to 1.031	Yes	*	0.0241

Table 8: P-values for SCB Medial Femoral Condyle Bone Volume

Discussion:

The unfortunate effects of osteoarthritis in day to day function are still an extremely prevalent issue, categorized by joint immobility, cartilage loss, structural changes, and pain. Our lab group has been persistent in working with a variety of therapeutics in different models, specifically preclinical rodent models. The implementation of therapeutic devices could help alleviate the unfortunate symptoms and permanent disability of OA, ultimately saving billions of dollars. In our particular study, we used only a select few therapeutics, therapeutics which had shown promising effects in various published papers aimed at targeting unresolved inflammation and protection against cartilage loss (Zhao et al., 2004), (Su et al., 2022), (Kieswetter et al., 1997). This highlights the need to broaden the scope of drugs administered to the knee, primarily because we saw varying degrees of significance across experimental groups.

In the scope of Ferulic Acid delivery, it is normally administered via oral gavage. This is primarily because it is an abundant antioxidant in the human diet and has beneficial properties when absorbed in the stomach. FA absorption and metabolism in rat stomachs and livers has been studied, the delivery of FA intraarticularly as a therapeutic has not (in as much depth) (Zhao et al., 2004). We administered FA intra articularly with the same method as the other therapeutic compounds. While injection isn't the primary mode of administration for FA, oral gavage could potentially create confounding variables in regard to pathological outcomes alongside the very feasible possibility that the FA doesn't get to the knee joint successfully. This would require further dosage analysis to make sure we are providing an appropriate amount of FA with no guarantee of effect and/or no guarantee of reaching the synovial capsule of the knee. Gastric absorption of FA could distribute the drug anywhere in the body, whereas direct injection guarantees its destination in the knee's synovial joint. The dosage of Ferulic acid via oral gavage

was 4.5µmol/mL (Zhao et al., 2004), whereas the dosage for our delivery was 77µmol/mL . We administered 30µL of FA at a concentration of 77µmol/mL to our rats intraarticularly (an injection of 2.31µMol FA); delivery of FA via oral gavage varied on the weight of each animal and was administered at a concentration of 8µmol/kg BW (Zhao et al., 2004). The weight of animals was not provided in the supplemental study, so assuming they were similar to the animals used in our study (300-340g), we can assume the amount of FA delivered to each animal via oral gavage was around 2.4µMol. Since administration in our study varied from typical procedures, comparing results is difficult. However, FA has been found to suppress IL1β induced degeneration of chondrocytes (Du et al., 2021), so administration of FA would hopefully show no cartilage loss, a trend of which we were able to observe in our own study using µCT Tomography.

Resolvin D1 is a potent lipid molecule that has shown previous promise for treating OA. It has been identified as an antiapoptotic, anti-inflammatory, and antioxidant compound that acts through various mechanisms, all of which can help reduce severity of OA (Su et al., 2022). RvD1 was found in this article to be able to successfully target OA induced fibroblast-like-synoviocytes (FLS) while having no cytotoxic effect on normal human FLS's; RvD1 arrested OA-FLS's in the G2 phase of cell differentiation, ultimately reducing mitosis and cell proliferation. Cell cycle arrest was induced via the phosphorylation of the protein YAP in the Hippo-YAP pathway, further implying that RvD1 can downregulate the expression of YAP via phosphorylation and reduce FLS pathogenesis and proliferation (Su et al., 2022). In another article, it was concluded that RvD1 strongly reduces the recruitment of osteoclasts (cells that break down bone) and also reduces the expression of various inflammatory biomarkers such as TNF-a, IL-1b, and Nitric Oxide (Benabdoune et al., 2017). Reducing osteoclast activity in the

knee joint would ultimately reduce bone resorption (breakdown and/or removal), potentially changing how SCB and trabecular bone in various compartments might respond to drugs such as RvD1. Relating these results to the results of our pilot study, a trend was observed of RvD1 potentially preserving cartilage, but being unsuccessful in preserving the SCB of both the femur and tibia. This raises the question of if the RvD1 we administered was able to arrest OA-FLS, or more simply, if it was able to interact with the Hippo-YAP pathway at all.

PDGF has been tested to show its effect on cartilage and bone cell proliferation and production. Results of studies show that chondrocytes (cells that create cartilage) can respond to PDGF with an increase in proliferation, alongside showing how PDGF initiates extensive proliferation of osteoblasts (Kieswetter et al., 1997). Relating these results to our study, when we administered PDGF to the knee, we did not find any significant evidence of increased osteoblast activity, because presumably increased osteoblast proliferation would result in more bone volume in the knee joint. There is a possibility of chondrocyte activation, however based upon the thicker cartilage.

In Vitro Testing:

Results *in vitro* showed inconclusive results. In attempting to measure NO activity to cytokine concentrations, there was no significance across treatment groups and no real observable difference. There is a strong possibility that the cytokines administered, TNF-α and IL1, have alternative functions in regard to inflammatory pathways. NO and Nitrite, while it has a prominent role in inflammation and the immune response, are not the only molecules involved. As such, there is a possibility we measured two variables that don't have a strong interaction with one another, or we measured two variables that do have

direct interactions with one another but that don't have consistent/significant differences and changes. In regard to measuring metabolic activity with the alamar blue assay kit, results were equally as inconclusive as the nitrite assay. There was no significance across treatment groups, however there were some slight observable differences with some outliers. Since alamar blue is light sensitive, there is a possibility the solution was exposed to light, altering the final product. Similarly, there is a possibility we measured variables that don't have strong interactions with one another and play other roles in regulation of the immune response. The same can be assumed with DNA content in the Cyquant DNA assay measurements.

In Vivo Testing:

For *in vivo* results of this study, we were able to observe trends we would expect as a result of the BioSeb dynamic weight bearing system. Animals post ACLr ended up bearing less weight on their injured limb at all time points, however they were able to gradually bear more weight on their injured limb as time progressed. Significance was noted for cartilage thickness between Sham and PDGF+Gel in the tibia alongside significance between Sham groups and PDGF, PDGF+Gel for the femur. While significance was found for these data groups, alongside others in SCB, it is important to recognize that the incorporation of historical ACLr data that was not conducted in this pilot study could have altered significance both negatively or positively. While this could potentially be due to the therapeutics administered, it has been found in previous studies for cartilage to thicken in early stages of OA in animal models due to swelling and hypertrophy (Cotofana et al., 2012) (Buck et al., 2010). Evidence for cartilage thickening in human models with OA remains up in the air. This ultimately results in an inconclusive finding,

as while we saw cartilage retention/thickening, it may not have been due to the therapeutics. This raises an important question, is the presence of thicker cartilage good, or could it potentially result in unforeseen hindrances? If the extra cartilage growth is large enough, or located in a specific area prone to excess movement, it can restrict joint movement, cause pain, or lead to the formation of "loose bodies" within the joint. A loose body is typically categorized as a small chunk of bone or cartilage that breaks off of the knee joint that continues to grow inside the joint. This can potentially limit movement if a loose body gets lodged between joint surfaces, ultimately resulting in pain, swelling, and difficulty straightening the knee (*Articular Cartilage Damage*, n.d.) Despite the potential negative effects of having an excess of cartilage, having more cartilage to protect the bone articulations of the knee is a very beneficial effect. Significant change of cartilage volume in the medial tibial plateau was also noted between Sham and RvD1, RvD1+Gel groups, alongside significance between historical ACLr data and RvD1, RvD1+Gel. This result leaves final conclusions for this study inconclusive, as while we saw trends of decreased loss of cartilage between Sham and RvD1, RvD1+Gel groups, previous studies have highlighted that cartilage thickening and an increase in volume is associated with cartilage degeneration; specifically, the cartilage swells due to tissue integrity and quality decreasing. Despite this, in the presence of RvD1, there was no significant loss of cartilage on the medial tibial plateau in regard to volume and thickness. This result is consistent with findings in other literature stating how RvD1 is a good therapeutic intervention for treating OA (Dravid et al., 2022b), however it brings up another important question. Were the effects we witnessed impermanent in both the realm of cartilage degradation and in cartilage regeneration?

We were able to observe an opposite trend in regards to bone degeneration; while therapeutics were effective in preserving/forming cartilage, they were unsuccessful in preserving

the layers of bone directly under the cartilage. Significance was found in bone volume mean/density in the medial tibial plateau between Sham and RvD1+Gel, PDGF, and Ferulic Acid, alongside significance found between historical ACLr data and all therapeutic groups. Significance in SCB bone volume changes were noted between Sham and RvD1+Gel and Ferulic Acid. These significant changes were depicted through loss of bone, contrasting the preservation of cartilage seen in the very same joints. Significant loss in trabecular bone in the tibia was also noted between Sham and RvD1+Gel groups alongside historical ACLr data and RvD1+Gel. A decrease in trabecular bone can ultimately lead to weaker bone and an increased likelihood of fractures, a result that most likely stemmed from variations in animal weight during the NIKI procedure. Similar effects of subchondral bone loss were seen in the medial femoral condyle; significance was noted for bone volume changes between Sham and most therapeutics (RvD1, RvD1+Gel, PDGF+Gel, Ferulic Acid) alongside historical ACLr data and the same previous therapeutics. Significant trabecular thickness data points were noted between Sham and RvD1, RvD1+Gel, PDGF+Gel, and Ferulic Acid while also seeing significance between historical ACLr data and RvD1. Similarly to losing cartilage, loss of bone can have similar effects to the knee joint that is typically seen in OA. The most prominent of these symptoms being pain, stiffness, reduced ROM, and instability. With the loss of bone (also seen in loss of cartilage), is the growth of bone spurs, commonly referred to as osteophytes. In OA, when cartilage deteriorates, bone on bone contact is expected. Physiologically, the body responds by producing new bone tissue around the joint margins to compensate for the damage, forming osteophytes. These newly formed osteophytes can further irritate the joint, causing even more pain and limiting movement (Hashimoto et al., 2002). Osteophytes are a very important marker of OA, alongside having a large a large role in the pain aspect of this disease. There is an important

relationship to be made in regards to therapeutic administration and reduction of osteophytes, in fact RvD1 has been found to have a very strong inhibitory effect on osteophyte growth and pain, making it a promising strategy to treat OA (Dravid et al., 2022).

The previously expanded upon outcomes from the *in vivo* section of this pilot study do not provide any clear results. While we did see preservation of cartilage, we were unable to see preservation of bone; therapeutic efficacy would most likely highlight preservation of both bone and cartilage and maintain them around values close to Sham. There were notable trends of significance surrounding the usage of RvD1, in both its free and microgel encapsulated form. RvD1 has been highlighted in many outside sources of literature to contain many anti-inflammatory properties and overall effectiveness in treatments of OA (Dravid et al., 2022a). For this reason, next steps include expanding upon the usage of RvD1 via dosage sweep (testing different concentrations of RvD1 to identify appropriate levels as opposed to using only one concentration with varying therapeutics). Similarly, we plan to administer RvD1 in a fixed interval across groups, with some groups getting more injections compared to others. Existing literature highlights the efficacy of prolonged administration of RvD1 to stabilize it *in vivo*, increase its availability in the joint for longer, and reduce the influx of inflammatory cells produced by cytokines such as neutrophils (Dravid et al., 2022b).

Conclusion:

Complications of Osteoarthritis are still a persistent issue in today's day and age, despite billions of dollars being allocated in an attempt to advance potential treatments. This emphasizes the need for further exploration in the realm of pathological changes of OA and how, if changed, lessening these symptoms can truly help an individual heal and return back to a physically normal life. Changes, particularly loss, of bone and cartilage in the intra articular space of the knee is a common if not cardinal pathological change of OA, of which is even more accelerated with a joint injury such as an ACL rupture. Overall, our therapeutic pilot study demonstrated various significant changes in regard to cartilage and bone changes, with some being more promising than others; similarly representing a good starting point for further research.

In vivo there were clear trends of bone and cartilage pathology progression with therapeutics implemented. All realms of cartilage in the medial aspects of both the femur and tibia showed the therapeutics were effective in preventing cartilage breakdown and loss. However, the exact opposite was found for the subchondral bone, where the therapeutics were ineffective in preserving bone integrity and showed clear bone loss. Whether or not these results could help alleviate OA symptoms is unclear.

Overall therapeutic administration into the knee with varying drugs showed similar pathological progression of Osteoarthritis. This ultimately shows therapeutic results were inconclusive in regard to changing OA pathology, thus highlighting the need to focus on a RvD1 dosage sweep in the hopes of seeing more distinct changes.

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