Creating an *In Vitro* Protocol to Analyze the Effects of Microgravity on Chondrocyte Metabolism: A Pilot Study

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Abstract

The effects of spaceflight on astronaut health have been of great importance for human space flight, and long-term space flight will require a better understanding of the physiological effect microgravity has on the human body. Limited research has been performed analyzing the effect of microgravity on articular cartilage (AC). AC is necessary for proper functioning of joints by providing smooth, almost frictionless surface for the joint to move on, and to cushion some loading the joint experiences. Due to altered loading of joints within a microgravity environment, injury and illness within joints may occur. Osteoarthritis (OA) is a debilitating, painful joint disease that is though to be caused by altered loading of joints. Therefore, joints within a microgravity environment, may develop OA-like symptoms and illnesses. In order to investigate the effect of microgravity on articular cartilage, encapsulated chondrocytes were subjected to 4-days of microgravity exposure. Global metabolomics of metabolite extractions from the encapsulated chondrocytes, and metabolite secretions from the chondrocytes were analyzed to study the effects of microgravity on chondrocyte metabolism.

Introduction

The effect of microgravity (10^{-6} g) on the human body is of great concern for the future of human space flight. Having evolved under 1g, humans' bodily functions have developed and often become reliant upon the constant presence of this force. Microgravity experienced from space flight has been shown to have detrimental effects on human health including neurovestibular problems, psychological problems, and

prominent effects on both the cardiovascular and musculoskeletal systems.[1, 2] The musculoskeletal system includes bone, skeletal muscle, cartilage, tendons, and ligaments, and has been subject to an immense amount of research in order to understand the effect of reduced loading from microgravity.[2] Microgravity causes reduced loading of the musculoskeletal system which has numerous detrimental impacts including demineralization of bone, loss of skeletal muscle mass and strength, atrophy of bone and muscle, and a higher risk for herniated disks and vertebral disk damage.[2, 3]

Most research regarding the effects of microgravity on the human musculoskeletal system has focused on bone and muscle, while there is relatively limited research on the effects of reduced loading from microgravity on articular cartilage. Articular cartilage (AC) is a connective tissue found within articulating joints that provides a smooth surface for joint movement and manages loads placed upon the joint.[4] Due to a lack of vascular tissue, AC has a limited ability to repair itself. Therefore, the health and maintenance of AC is important for lasting joint health.[4]

Articular Cartilage consists of an extracellular matrix (ECM) with a low concentration of chondrocytes distributed throughout.[4] The ECM primarily consists of water, collagen, glycoproteins, proteoglycans, and many other noncollagenous proteins. Within the ECM, chondrocytes are surrounded by a thin pericellular matrix (PCM).[5] Different than the ECM, the PCM is characterized by type VI collagen and perlecan. The physiological role of the PCM is still being analyzed, but it is currently thought to be the main interface where biochemical signals and biomechanical signals are transduced. While the ECM is primarily thought of as a structural matrix that is important for overall cartilage structure and function, the PCM may be more important for regulating the

mechanobiology and mechanical environment for the chondrocytes themselves. The PCM plays its own, distinct, and important role in cartilage function.

Chondrocytes are highly specialized, mechanosensitive cells that help maintain the PCM and ECM. Regular loading of articular cartilage stimulates chondrocytes to promote anabolism to rebuild and strengthen the cartilage.[6, 7] Overloading and consistent unloading of AC can disrupt this balance of anabolism and catabolism, leading to the breakdown of the AC due to an increase in catabolic activity.[3, 6, 7] Specific effects of unloading on AC include: decrease of proteoglycan content and synthesis, reduced cartilage stiffness, collagen II degradation, cytoskeletal reorganization, decreased ECM protein production, ECM mineralization, decreases in aggrecan content, thinning of articular cartilage, and ECM degredation.[3 ,6 ,8 ,9]

Osteoarthritis (OA) is also similarly characterized by collagen II degradation, proteoglycan loss, cytoskeletal reorganization, and reduced cartilage stiffness.[6, 8, 9, 10, 11] This form of arthritis is the most common form of joint disease across the globe, with 10% of the population over 60 years of age affected by OA.[10, 11] Symptoms of OA include joint stiffness, joint pain, loss of joint function, and a poorer quality of life. [9, 11, 12] Current research also suggests that OA may result from an imbalance of catabolic and anabolic activities within the joint.

Since microgravity significantly reduces the mechanical loads applied to AC, long term spaceflights could possibly increase the risk of AC degradation and the subsequent development of OA. [3, 5, 6, 7, 9, 13]. The degradation of AC during spaceflight may pose a significant problem for space travel. Not only is the development of joint disease (OA)

post-spaceflight a considerable concern, but the loss of AC during spaceflight could be detrimental to an astronauts' abilities to perform vital tasks during spaceflight. Astronauts on the International Space Station must perform rigorous tasks that require great strength and mobility. The pain and mobility loss usually associated with cartilage degradation would inhibit astronauts from performing their duties to the best of their abilities. Similarly, reentry into the Earth's atmosphere also applies an immense physical toll on the human body, and with an all-around weakened musculoskeletal system, astronauts are at a greater risk for injury upon reentry. Therefore, keeping AC healthy and properly functioning is a key component of keeping the musculoskeletal system of astronauts healthy and functional for duties in space, for reentry into atmosphere, and to prevent long term damages.

The abnormal joint loading experienced in microgravity environments may change the metabolism of chondrocytes and stimulate a catabolic response to the reduced mechanical load. Understanding this possible effect of microgravity on chondrocyte metabolism may prove valuable in keeping astronauts healthy during and after space flight.

In order to expand our understanding of microgravity and chondrocyte mechanotransduction, the goals of this study are: (1) To develop and troubleshoot an *in vitro* protocol to expose encapsulated chondrocytes to microgravity; (2) Analyze the metabolic activity of encapsulated chondrocytes when exposed to microgravity as compared to those under normal gravitational conditions. The metabolic response of chondrocytes to microgravity should provide a clearer picture of overall chondrocyte mechanotransduction and its implications for overall joint health.

Methods

Cell Culture:

Human chondrocytes (SW1353) were purchased from the American Type Culture Collection (HTB-94). The frozen vial was thawed in a water bath at 37°C with gentle agitation. Complete growth medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1 % Penicillin-Streptomycin was also warmed to 37°C in the water bath. Sprayed with 70% ethanol, the vial was then moved into a laminar flow hood where strict aseptic technique was followed. Vial contents were emptied into a 50 mL falcon tube with 9 mL of complete media. The tube was centrifuged at 125rcf for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 12 mL of complete growth media and added to a petri dish. The cell culture was incubated at 37°C, 5% CO_2.

Cell cultures were maintained and split at 70% confluency. The media was aspirated from the petri dish and cells were washed with 1X PBS solution twice before 5 mL of warmed Trypsin (37°C) was added to the petri dish. The petri dish was then placed into an incubator (37°C, 5% CO_2) for 15 minutes. After 15 minutes, cells were checked under a microscope to confirm that they had detached from the surface of the petri dish, and 10 mL of complete growth media was added. Contents of the petri dish were pipetted into a 50 mL falcon tube. Tubes were centrifuged at 300 rcf for 5 minutes. Supernatant was aspirated off and the pellet was resuspended in 36 mL of media. After resuspension, 12 mL of media and cells were pipetted into 3 petri dishes. The petri dishes were checked under a microscope to confirm the presence of cells, and then placed in an incubator (37°C, 5% CO_2).

Gel Encapsulation:

All water, agarose, and other equipment used for this procedure was sterilized in an autoclave. First, 15 mL falcon tubes were warmed in a 37°C water bath. The 4.5% w/v agarose was made by microwaving for a total of 30 seconds and stirring in every 10 seconds. The agarose solution was then poured into the pre-warmed 15 mL tubes—9 mL into one tube. In the tube with 9 mL of agarose, 1 mL of complete growth media with 1 x 10^6 cell count was added. Wells in a 96-well plate were filled with the agarose mixture to encapsulate the cells and create the cartilage constructs. Once the gels cooled, a razor blade was scraped across the top of the well plate, and the cartilage constructs were removed using a small weighing spatula.

RCCS Initiation:

Complete growth media was added to the rotary cell culture system (RCCS) vessels through the fill port, then a cartilage construct was added through the fill port. To remove all bubbles from the RCCS vessels, a 5 mL syringe with complete growth media was added to one port, then an empty syringe was added to the other. The bubble was positioned under the empty syringe and was pulled into it by simultaneously pushing media into the RCCS vessels and pulling the bubble out with the empty syringe. The RCCS vessels were attached to the RCCS motor inside an incubator (37°C, 5% CO_2). The RCCS was turned on and rpm was determined manually to position the cartilage construct in constant free fall. Agarose constructs left within media in a non-rotating 140 mL culture flask were used as the controls. Both experimental constructs in the RCCS

vessels and control constructs were maintained either under simulated microgravity or normal gravitational forces for a period of four days.

RCCS Culture Maintenance:

Each day, the RCCS was monitored for bubble formation and to ensure the constructs remained in a state of microgravity. When bubbles were observed within a vessel, the RCCS vessel was removed while the motor was running to keep other constructs in a state of microgravity. Bubbles were removed in the same manner detailed above. When cartilage constructs fell out of a microgravity environment, rpm was adjusted until all four constructs were experiencing microgravity.

Metabolite Extraction:

At the end of the fourth day, cartilage constructs and media were removed from the experimental vessels and control culture flasks, and extracted using an established protocol within the Alyssa K. Hahn lab.[14] A 1 mL sample of media was removed from the vessels, and the cartilage constructs were homogenized in a 3:1 methanol:water solution. The homogenate and the media samples were centrifuged at 13523 rcf for 10 minutes. The supernatant was removed into separate microcentrifuge tubes with a 80:20 methanol:water solution and stored at -20 C for 30 minutes. Samples were vortexed for one minute then centrifuged at 16100 rcf for 5 minutes. Supernatant was dried down in a vacuum concentrator. After being dried down, proteins were precipitated from the samples with 1:1 acetonitrile:water solution at -20 C for 30 minutes, then vortexed for 1 minutes. Samples were centrifuged at 16100 rcf for 5 minutes, then the supernatant was dried down in a vacuum concentrator a second time. Metabolite extract samples were

stored at -80 C until liquid chromatography mass-spectroscopy (LC-MS) was performed at MSU in collaboration with the June Lab.[15]

LC-MS: Metabolite extracts were resuspended in 50:50 water:acetonitrile solution as the LC-MS injection solution. The LC-MS system used for analysis was an Agilent 1290 UPLC connected to an Agilent 6538 Q-TOF mass spectrometer. Separation of metabolites occurred on a Cogent Diamond Hydride HILIC 150 x 2.1 mm column using an optimized normal phase gradient elution method.

Live Dead Staining:

Experimental RCCS vessels and control cultures flasks were transported to MSU for live dead staining and confocal imaging. Constructs were removed from their respective containers and placed in a solution with 8µmol calcein and 75 µmol propium iodide. Samples were incubated within this solution for 30 minutes then examined under a confocal microscope to confirm the presence of alive and dead cells within the cartilage constructs. Live cells were dyed green while red cells were dyed red.

Metabolomic Analysis with MetaboAnalyst:

The software MetaboAnalyst was used for statistical analysis of the LC-MS data.[16] Data was split into 4 main cohorts: experimental construct metabolites, experimental metabolite secretions, control construct metabolites, and control metabolite secretions. Three different comparisons were made between the different cohorts. One comparison analyzed the metabolic differences between experimental construct metabolites and control construct metabolites, while another comparison analyzed the

metabolic differences between experimental metabolite secretions and control metabolite secretions. All construct metabolites were compared against all metabolite secretions to identify differences between metabolites found within encapsulated chondrocyte metabolites and their metabolic secretions.

For each comparison, all metabolite features with a median of zero across all cohorts were removed, and all values of zero were replaced with NAs. Within MetaboAnalyst, all NAs were replaced with 1/5 the smallest size with the data set and there was no data filtering performed. For normalization, data were log transformed and the data were auto-scaled. Data was checked to ensure a standard bell-curve was present. Univariate, multivariate, and clustering analyses were then performed within MetaboAnalyst.

T-test, volcano plot analyses, principal component analyses (PCA), partial least-square analyses (PLS-DA), dendrogram analyses, and heatmap analyses were performed using MetaboAnalyst. T-test analyses used a false discovery rate (FDR) of 0.05 as the p-value threshold. Volcano plot analyses used a direct comparison, the raw p-value of 0.05 as the p-value threshold, and a fold change threshold of 2.0. Dendrogram analyses were performed with a Euclidean measuring distance and the Ward clustering algorithm, and heatmap analyses were performed with Euclidean measuring distance and the Ward clustering algorithm as well.

Peaks-to-Pathway analyses using the mummichog algorithm, a molecular weight balance of 5.0 ppm, p-values, and in positive mode were also performed using MetaboAnalyst. Significant metabolite features from t-test analyses and from volcano

plot analyses were both used for peaks-to-pathway analysis. The upregulated and downregulated significant metabolite features discovered from volcano plot analyses were analyzed separately. The human (KEGG) pathway library was used to identify metabolic pathways and metabolite compounds.

Results and Discussion

Live Dead Staining and Confocal Microscopy:

In control constructs, live and dead cells were found using confocal microscopy (**Figure 1A-D**). This confirms that the protocol developed throughout this experiment was successful at encapsulating chondrocytes, and it also confirms that chondrocytes can survive within these cartilage constructs for at least four days under normal gravitational forces. Live and dead cells were also found to be present within cartilage constructs after a four-day exposure to a simulated microgravity environment—confirming that encapsulated chondrocytes can survive four-days within simulated microgravity **(Figure 1A-D)**.

While live and dead cells were both found present within the agarose chondrocytes, there were not enough cells present to perform a reasonable statistical analysis using the confocal microscopy. Thus, confocal microscopy was only used within this study to confirm the presence of live chondrocytes within the control and experimental constructs. The lack of chondrocytes found suggests that increasing the concentration of cells during the encapsulation protocol is of extreme importance for future studies to ensure the possibility of more thorough confocal microscopy analysis.

Throughout this protocol, many unstained cells were observed. This could possibly be due to the concentrations of stain used, or the properties of the cartilage construct restricting the chondrocytes from being stained. Future research and studies using this protocol could attempt to solve this issue to have more accurate confocal imaging results. Solving this issue might result in enough chondrocytes within the agarose gels for statistical analysis of the confocal imagery without an increase in concentration of chondrocytes during the encapsulation protocol.

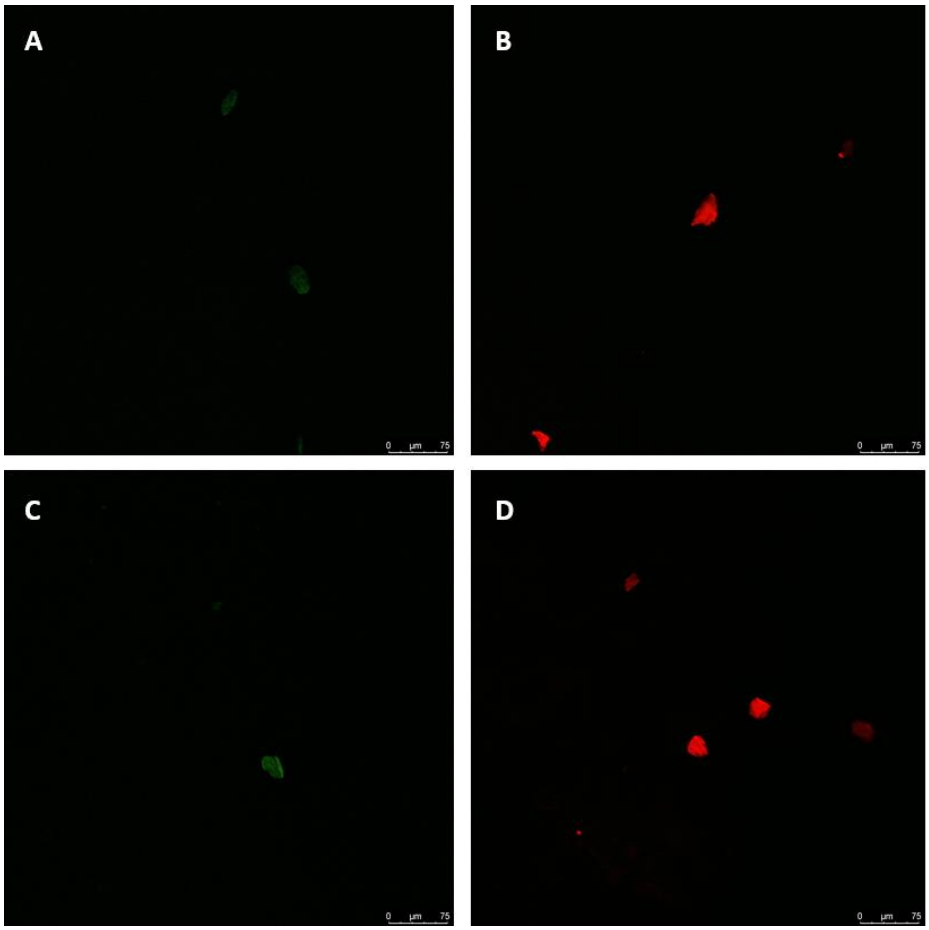

Figure 1: Images from confocal imaging of encapsulated chondrocytes within 4.5 % w/v agarose. (A) Live cells within an agarose construct after 4 days of microgravity exposure. (B) Dead chondrocytes within a control agarose construct. (C) Live chondrocytes within a control agarose construct. (D) Dead chondrocytes within a cartilage construct after 4-days of microgravity exposure.

Metabolomic Analysis:

Statistical analysis investigated the three comparisons outlined in the methods:

(1) All Metabolites from Cartilage constructs vs All Metabolite Secretions Extracted from Media:

T-test analysis of this comparison found 1175 significant metabolite features between all cartilage constructs and all metabolite secretions (**Figure 2A**). Volcano plot analysis found 384 statistically significant downregulated metabolite features, and 386 statistically significant upregulated features (**Figure 2B**). Principal component analysis (PCA) and partial least square (PLS-DA) analyses show distinct separation between metabolites from agarose gel constructs and secreted metabolites extracted from the media, with PLS-DA showing a notably higher degree of separation due to PLS-DA being a more supervised from of clustering analysis (**Figure 2C-D**). Heatmap and dendrogram analyses cluster samples revealed partial clustering of samples from the same cohorts (**Figure 3A-B**).

These analyses illustrate that metabolites found within the encapsulated chondrocytes vary immensely compared to the metabolites secreted by the chondrocytes. Secreted metabolites are of great importance when studying chondrocyte metabolism, as chondrocyte secretions are what maintain and alter the ECM of AC. Additionally, secreted metabolites may be detected as biomarkers to indicate AC degradation in response to microgravity exposure. It is important to investigate construct metabolites from the chondrocytes and secreted metabolites found within the media to understand a

more comprehensive picture of changes within the metabolomes of encapsulated chondrocytes.

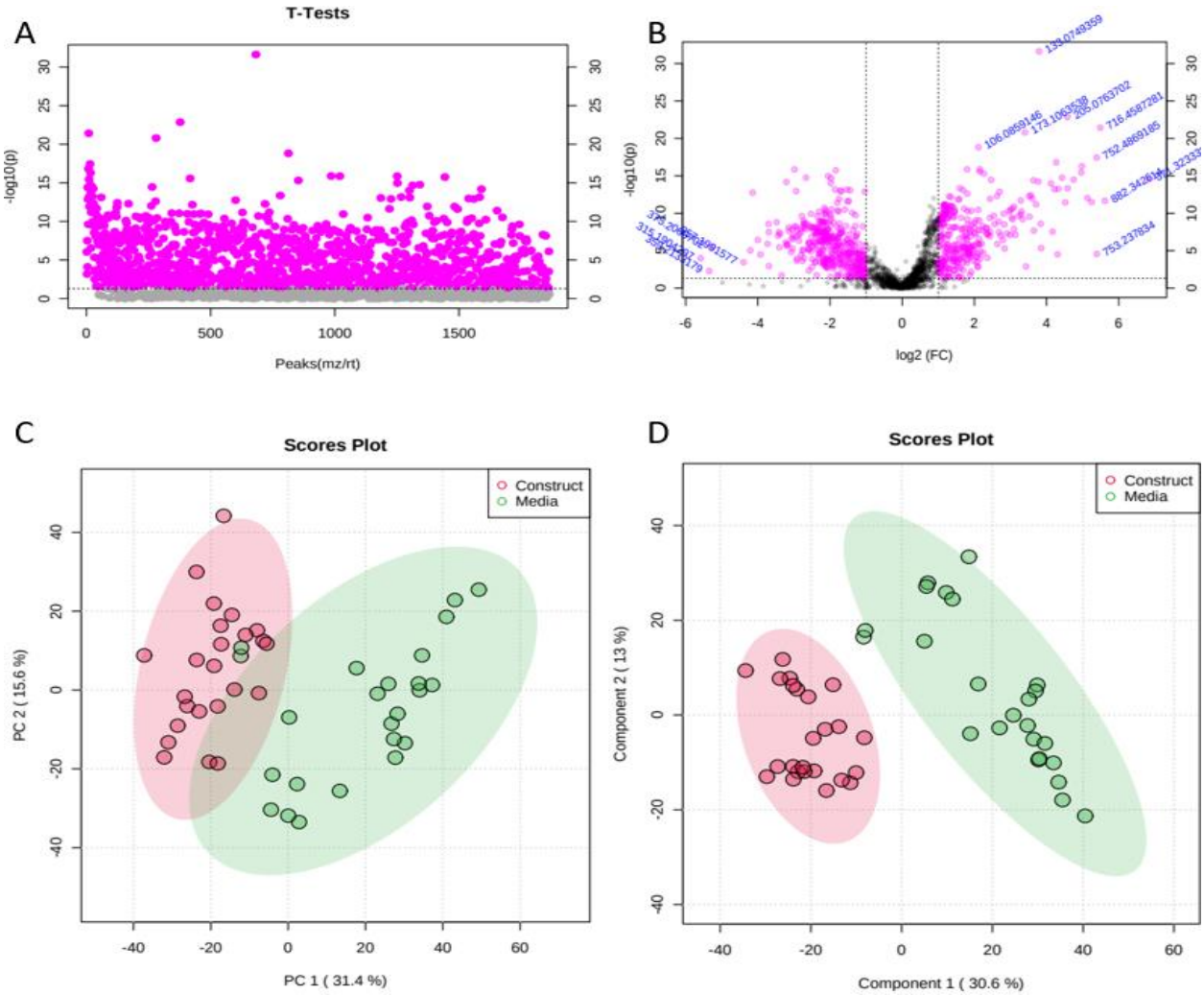

Figure 2: Univariate and multivariate analysis of comparison (1), all metabolites from cartilage constructs vs all metabolite secretions extracted from media. (A) T-test analysis where all pink points are metabolite features with a FDR value below 0.05. (B) Volcano plot analysis where all pink points are metabolite features with a p-value below 0.05. The labels on these points are their specific m/z values from LC-MS analysis. Significant metabolites to the left are downregulated while significant metabolites to the right are upregulated. (C) PCA analysis using the two principal

components that show the most variance between the two cohorts. (D) PLS-DA analysis using the two principal components that show the most variance between the two cohorts**.**

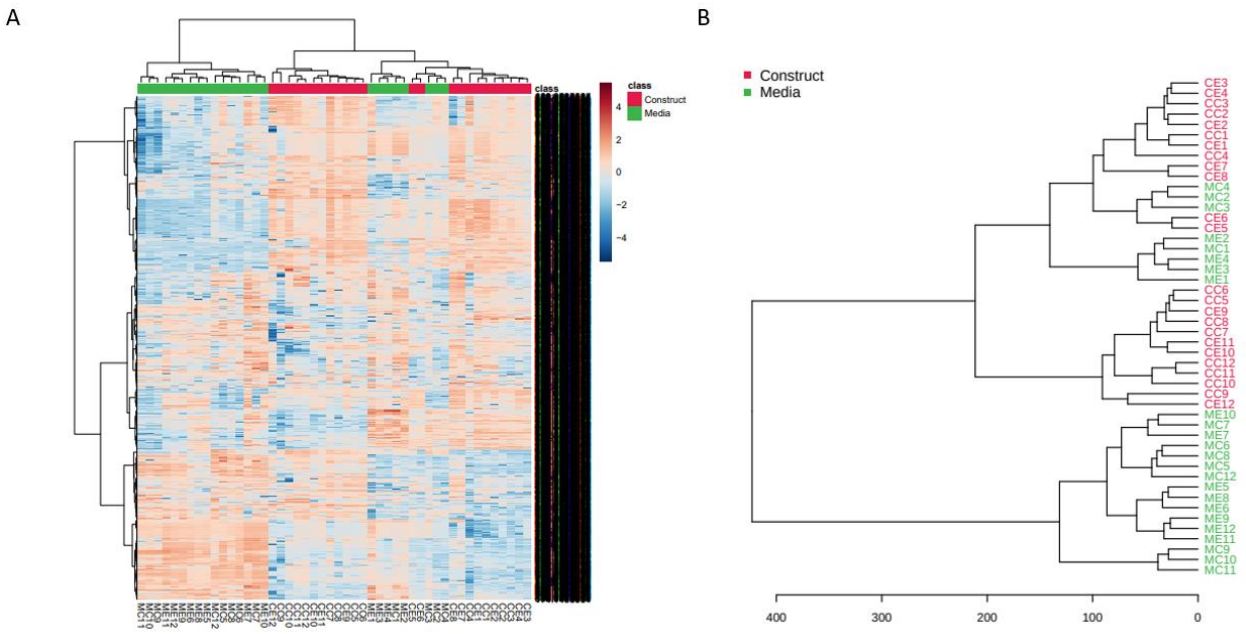

Figure 3: Clustering analysis of comparison (1), all metabolites from cartilage constructs vs all metabolite secretions extracted from media. (A) Heatmap analysis where blue are downregulated metabolite features and red are upregulated metabolite features. In the x-axis, green samples are secreted metabolites extracted from media and red samples are metabolites extracted from encapsulated chondrocytes. (B) Dendrogram analysis where green samples are secreted metabolites extracted from media and red samples are metabolites extracted from encapsulated chondrocytes.

(2) Experimental Cartilage constructs vs Control Cartilage constructs

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T-test analysis comparing metabolites extracted from experimental cartilage constructs versus control cartilage constructs found 50 significantly different metabolite features (**Figure 4A**). Volcano plot analysis found 60 statistically significant

downregulated metabolite features and 60 statistically significant upregulated metabolite features (**Figure 4B**). PCA analysis showed little to no separation between the cohorts, while PLS-DA was able to show separation between the cohorts in three dimensions (**Figure 4C-D**). Similarly, clustering analysis was not successful in clustering samples within their respective cohorts (**Figure 5A-B**). There were not enough significantly different metabolites discovered with t-test analysis, nor with volcano plot analysis, to perform peaks to pathway analysis with the comparison of these two cohorts.

Metabolites from encapsulated chondrocytes do not appear to be significantly altered when placed within a microgravity environment for four-days. This suggests that short term space flight and microgravity exposure may not have a detrimental effect on AC. However, more data should be collected to confirm this result, and future research should focus on increasing sample size and lengthening the exposure to microgravity.

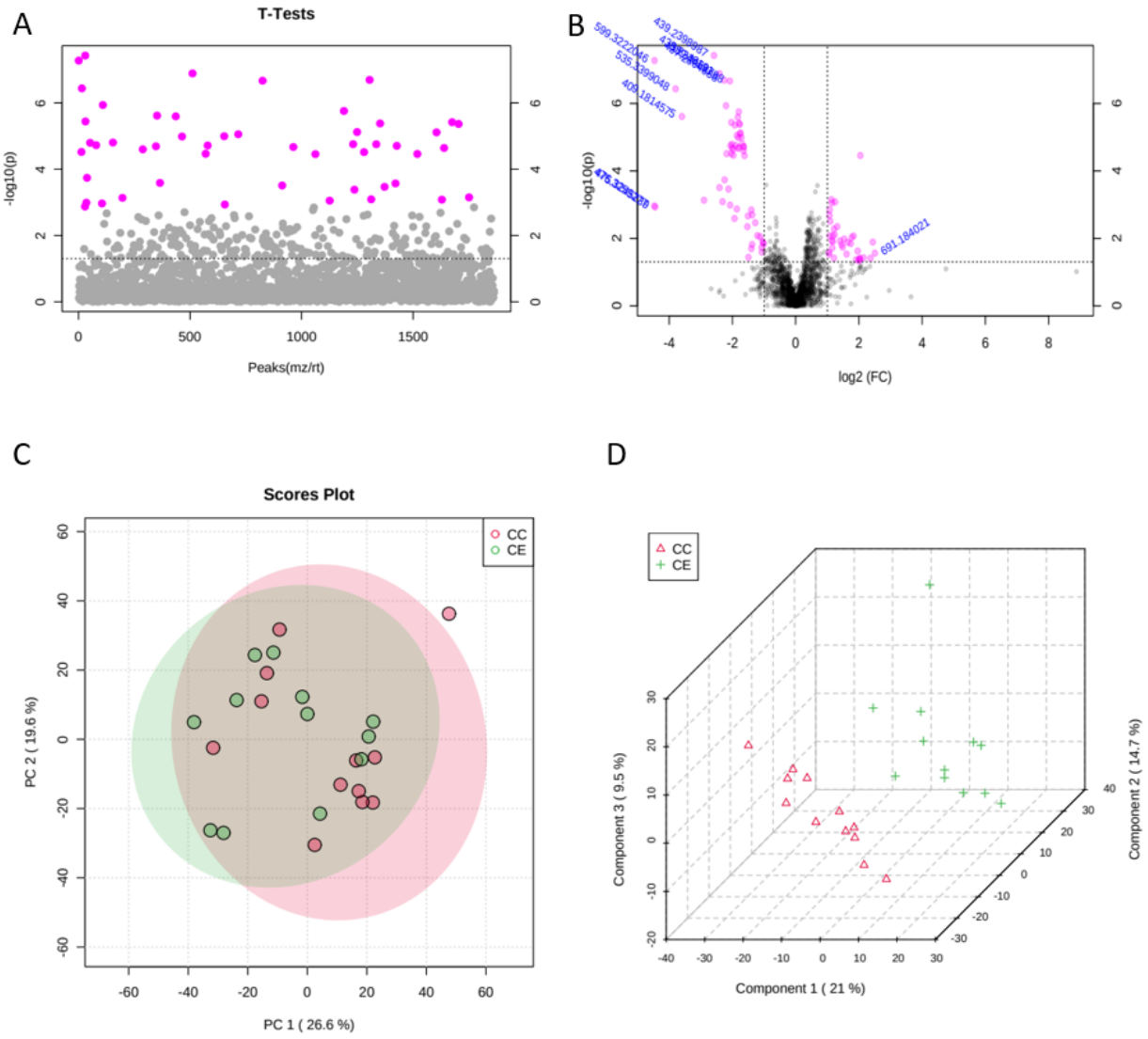

Figure 4: Univariate and multivariate analysis of comparison (2), experimental cartilage constructs vs control cartilage constructs. (A) T-test analysis where all pink points are metabolite features with a FDR value below 0.05. (B) Volcano plot analysis where all pink points are metabolite features with a p-value below 0.05. The labels on these points are their specific m/z values from LC-MS analysis. Significant metabolites to the left are downregulated while significant metabolites to the right are upregulated. (C) PCA analysis using the two principal components that show the most variance between the two cohorts. (D) PLS-DA analysis using the three principal components that show the

most variance between the two cohorts. Separation was not observed with two principal components but is shown with three**.**

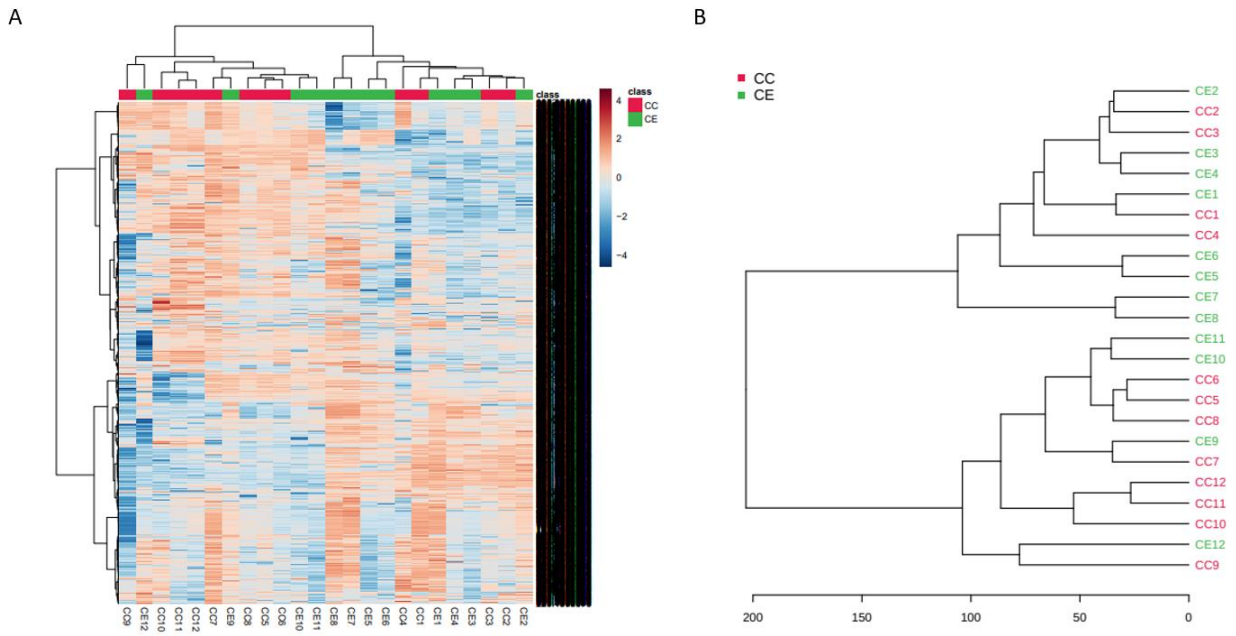

Figure 5: Clustering analysis of comparison (2), experimental cartilage constructs vs control cartilage constructs. (A) Heatmap analysis where blue are downregulated metabolite features and red are upregulated metabolite features. In the x-axis, green samples are metabolites extracted from encapsulated chondrocytes after four days of exposure to microgravity and red samples are metabolites extracted from encapsulated chondrocytes after four days of control conditions. (B) Dendrogram analysis where green samples are metabolites extracted from encapsulated chondrocytes after four days of exposure to microgravity and red samples are metabolites extracted from encapsulated chondrocytes after four days of control conditions.

(3) Experimental Secreted Metabolites from Media vs Control Secreted Metabolites from Media

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T-test analysis comparing secreted metabolites extracted from media after four days of microgravity exposure to secreted metabolites extracted from media after four days of control conditions found 50 significantly different metabolite features (**Figure 6A**). Volcano plot analysis found 110 statistically significant downregulated metabolite features and 110 statistically significant upregulated metabolite features(**Figure 6B**). PCA analysis did not successfully separate the two cohorts, while PLS-DA was able to successfully separate the two cohorts (**Figure 6C-D**). Clustering analysis using dendrogram and heatmap analysis had similar results to comparison (2)—many samples are clustered with samples from a different cohort (**Figure 7A-B**). Also similar to comparison (2), volcano plot analysis and t-test analysis did not find enough significantly different metabolite features to run a peaks to pathway analysis of this comparison.

Metabolites from chondrocyte excretions do not appear to be significantly altered when placed within a microgravity environment for four days; suggesting that short term space flight and microgravity exposure may not have a detrimental effect on AC. However, this is only using secreted metabolites, and more data should be collected to confirm this result. Future research should focus on increasing sample size and lengthening the exposure to microgravity. Extracting and analyzing secreted metabolites from media also opens the door for future experiments to investigate a changing metabolome by taking media samples throughout a microgravity experiment at different

time steps. This would be important for experiments investigating longer periods of microgravity exposure.

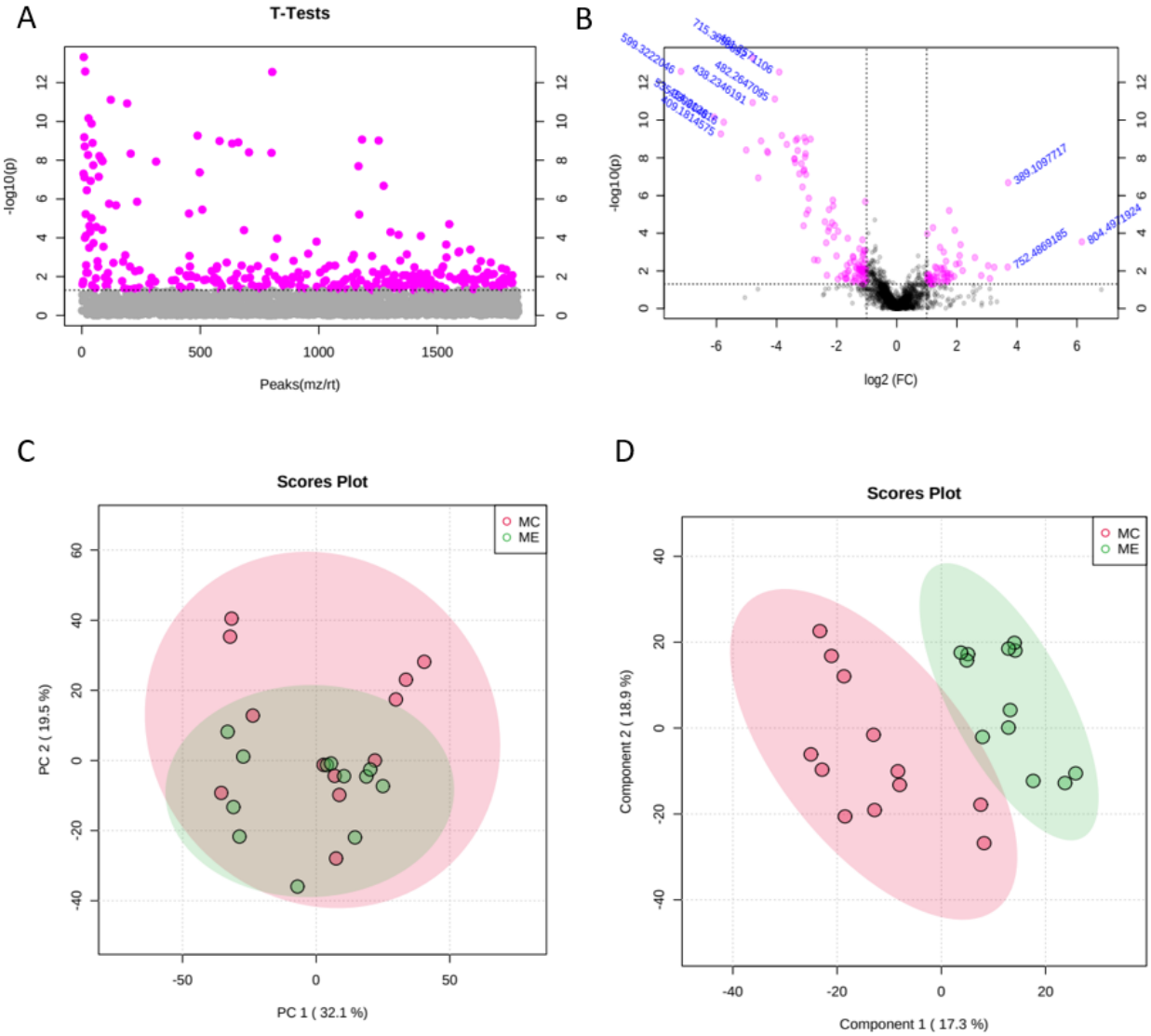

Figure 6: Univariate and multivariate analysis of comparison (2), experimental secreted metabolites from media vs control secreted metabolites from media. (A) T-test analysis where all pink points are metabolite features with an FDR value below 0.05. (B) Volcano plot analysis where all pink points are metabolite features with a p-value below 0.05. The labels on these points are their specific m/z values from LC-MS analysis. Significant metabolites to the left are downregulated while significant

metabolites to the right are upregulated. (C) PCA analysis using the two principal components that show the most variance between the two cohorts. (D) PLS-DA analysis using the three principal components that show the most variance between the two cohorts.

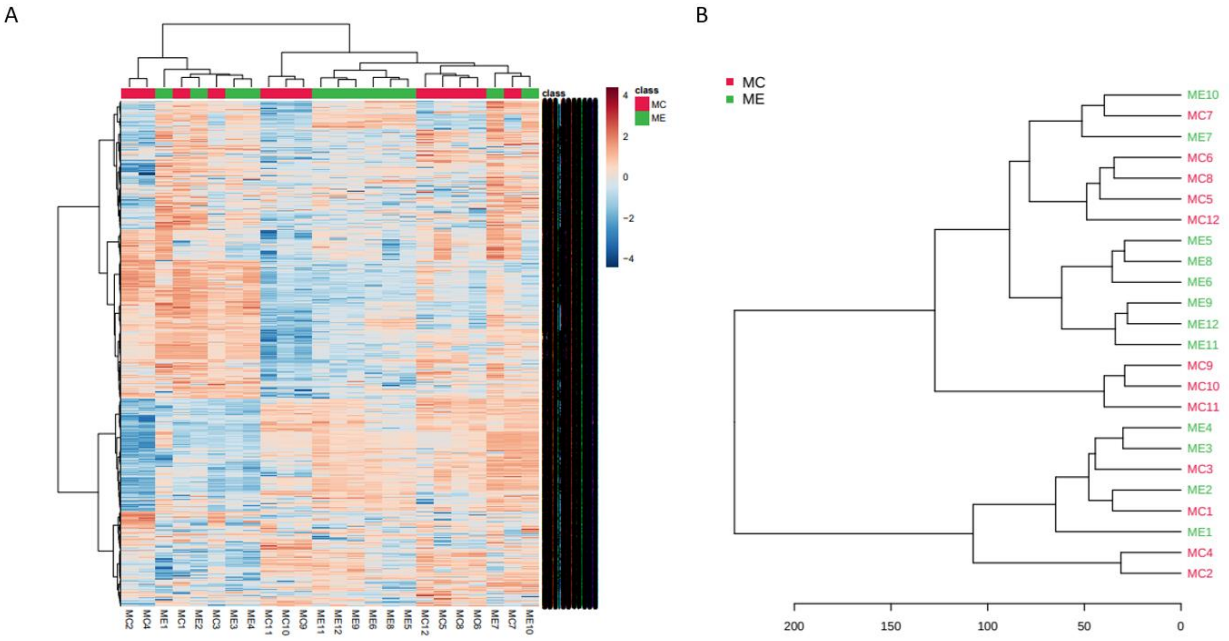

Figure 7: Clustering analysis of comparison (2), experimental cartilage constructs vs control cartilage constructs. (A) Heatmap analysis where blue are downregulated metabolite features and red are upregulated metabolite features. In the x-axis, green samples are metabolites secretions extracted from media after four days of exposure to microgravity and red samples metabolites secretions extracted from media after four days after four days of control conditions. (B) Dendrogram analysis where green samples are metabolites secretions extracted from media after four days of exposure to microgravity and red samples metabolites secretions extracted from media after four days after four days of control conditions.

Conclusion

This experiment began with two distinct goals: (1) To develop and troubleshoot an *in vitro* protocol to expose encapsulated chondrocytes to microgravity, and (2) Analyze the metabolic activity of microgravity-exposed encapsulated chondrocytes in comparison to those under normal gravitational conditions.

As a pilot study, the first goal was the priority, and it was completed successfully. Chondrocytes were successfully encapsulated within a cartilage construct, exposed to a microgravity environment, and metabolites were successfully extracted from the encapsulated chondrocytes as well as secreted metabolites from the media after microgravity exposure. To our knowledge, this is the first study to introduce and maintain encapsulated chondrocytes within a microgravity environment, and the first study to analyze the metabolomes of encapsulated chondrocytes and their secretions after *in vitro* exposure to microgravity.

While a successful protocol was developed, details within the protocol should be adjusted for future research. Increasing the concentration of chondrocytes initially encapsulated should provide cartilage constructs with a higher density of chondrocytes, and thus would enable better confocal microscopy results and increase the yield of metabolites extracted from constructs. More experiments should be performed to collect more data for analysis. Analyses looking into longer periods of exposure to microgravity are important for future research to accurately model current space flight durations and would be helpful in modeling longer space flight missions.

Using an RCCS to study *in vitro* cell culture in a microgravity environment has a few limitations. The biggest limitation is that the RCCS vessel had to be removed from the system to remove bubbles created during the experiment. Even if this problem is solved, for longer periods of microgravity exposure the vessels would still have to be removed from the system to replace media. The RCCS motor used in this experiment also only had one speed setting for all RCCS vessels. This single-speed option removed the ability to set different vessels at different rpm, so not every construct could exist in a perfect microgravity state. Because there were rare cases where it was impossible to keep all cartilage constructs within a perfect microgravity state, some cartilage constructs could have experienced forces that other constructs did not.

Regarding the second goal of the study, metabolomes from microgravity-exposed encapsulated chondrocytes were successfully compared against encapsulated chondrocytes exposed to normal gravitational conditions. The data collected indicate that short term exposure to microgravity does not significantly affect the metabolism of encapsulated chondrocytes. However, the preliminary data from this study are not sufficient for making a formal conclusion of the effects of microgravity on chondrocyte metabolism. Additional data, along with longer periods of microgravity exposure, are required to make a significant claim.

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