Effect of stretch and release on myofascial stem cell function *in vitro*: A putative model to understand the molecular benefits of the myofascial release (MFR) technique

Ben Smith, Shahnawaz Notta and Debasis Mondal
DeBusk College of Osteopathic Medicine, Lincoln Memorial University, Knoxville, TN 37932

Abstract

Background and Significance: Despite the beneficial effects of osteopathic manipulative techniques (OMT), there is a lack of *in vitro* models to understand the molecular mechanisms associated with these time-tested therapies. The Myofascial Release (MFR) technique is a non-invasive approach that involves passive stretching, hold and release, of the soft tissue to achieve myofascial homeostasis. Tissue-resident mesenchymal stem cells (MSC) can regulate the myofascial microenvironment by altering their secreted factors following stretch and release. Therefore, we initiated studies to develop an *in vitro* model to investigate the possible effects of stretch and release on MSC function, i.e. proliferation and differentiation capabilities, and changes in secreted factors. Preliminary optimization of protocols towards MSC culturing on a stretchable silicone membrane and their mechanical manipulation using the MechanoCulture FX (MCFX) machine, a programmable stretch and release device, is presented below.

Objective: To develop an *in vitro* model of MFR and examine the effects of stretch and release on MSC function and on their secreted factors (secretome).

Materials and Methods: General tissue culture supplies, DMEM media, and fetal bovine serum (FBS) were purchased from Fisher Scientific (Waltham, MA). MSCs from both male and female donors, stem cell growth and differentiation media, and staining solutions, were purchased from Obatala Biosciences (New Orleans, LA). The stretchable silicone culture membranes and the MCFX device were purchased from CellScale Biolabs (Ontario, Canada). The CCK8 dye, Crystal violent stain, and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Stem cell culture and differentiation assays were carried out according to the manufacturer's protocols. To compare MSC function under both static and stretched conditions, preliminary experiments were started to optimize MSC adhesion and growth on the silicone membrane. The MCFX device was programmed to mimic the MFR technique. Baseline proliferation and differentiation of MSCs under static conditions were also carried out.

Results: We were able to optimize the sterile technique of MSC growth on the MCFX device. We were also able to program the MCFX device to generate an amplitude of 3.6 mm stretch for 2 s, a hold duration of 1 s, followed by release. This cycle was repeated multiple times in the incubator. The silicone membranes had to be coated with poly-L-lysine to increase MSC adhesion to the substratum, especially following multiple bouts of stretch and release. Both CCK8 staining (proliferation) and crystal violet staining (cell imaging) showed MSCs growing on the silicone membrane and remaining attached following stretch and release. MSCs grown under static conditions differentiated to either adipocytes or osteocytes and could be stained with either Oil red-O or Alizarin red dyes. Experiments on MSC proliferation and differentiation under stretch condition is currently underway and data comparing static and stretch will be presented.

.

Introduction

Overview: Many health disorders have been reported to benefit from osteopathic manipulative methods (OMT), but the molecular mechanisms behind these treatments are still poorly understood. The Myofascial Release (MFR) technique is a non-invasive method for achieving myofascial homeostasis that involves passively stretching, holding, and releasing the soft tissue. By changing the substances they release after being stretched and released, tissue-resident mesenchymal stem cells (MSCs) can control the myofascial milieu. As a result, the goal of this work is to create an in vitro model to examine the potential impacts of stretch and release on MSC function, including their capacity for proliferating and differentiating as well as changes in secreted proteins. In vitro mechanical analysis of stem cells using mechanoculture devices has emerged as a promising approach for understanding the mechanical properties and response of these cells. Mechanoculture devices are specialized systems that are designed to apply mechanical stimuli to cells in a controlled and repeatable manner. These devices can be used to investigate a wide range of mechanical stimuli, including stretch, strain, compression, and shear stress. In the context of stem cell research, mechanoculture devices are often used to study the effect of mechanical forces on stem cell differentiation, proliferation, and migration.

Research Aim: The purpose of this work is to create an in vitro MFR model and investigate how MSC function and their secreted components are affected by stretching and releasing them (secretome). In order to compare the effects of stretch and release on MSC proliferation, differentiation, and alterations in their secretome, future studies evaluating the underlying processes of MFR and how it affects MSCs may build on the findings of this work.

Osteopathic Aim: This work simulates the effects of MFR on tissue-resident stem cells and their reactions to the tissue microenvironment in an effort to clarify how the MFR technique accomplishes its well-known positive effects in vivo. The creation of an in vitro model to study how MFR affects stem cell function may shed light on the application of osteopathic methods.

Methods

ASC culture and trypsinization: ASC were grown for 7 days in a T-75 flask until confluent. They were then washed with 3 ml of trypsin which was removed and another 4 ml of trypsin was added and placed at 37°C in the incubator for 1 minute and 45 seconds.

Trypsin was inactivated with 11ml of DMEM with 10% FBS for a total volume of 15ml and centrifuged leaving an ASC pellet. A total of 14ml of the solution was removed, and 7ml of StromalQual media was added to resolubilize the ASC pellet.

ASC growth on MCFX culture plates. Silicone wells used in the MechanoCulture FX (MCFX) device underwent a sterilization process with 70% isopropyl alcohol for 30 minutes, and then placed under UV lighting for 15 minutes. The wells were then rinsed with 350ul of Phosphate buffer solution three times. The wells were then coated with 250ul of 0.01% Poly-L-Lysine except the first column and left to dry overnight to increase ASC adherence to the well. Poly-L-Lysine solution was then removed the following day, and 250ul of the ASC in StromalQual media was added to each silicone well leaving the first column as a control and placed at 37°C in the incubator for 24 hours and the Mechanoculture FX program was initiated. In a previous experiment Poly-L-Lysine was left for four hours however, it did not produce the adherence we needed in order to observe a result from a stretch model.

Crystal violet staining of ASCs. The media was removed from the silicone wells and 300ul of 30% crystal violet was added to column one and incubated for 15 minutes at 37°C and then removed, discarding the crystal violet in column one. Under microscopy increased adherence compared to four hours of Poly-L-Lysine coating was observed. The remaining wells then were stained with 20% CCK8 and placed in the incubator at 37°C for three hours then removed. The supernatant from four of the Poly-L-Lysine silicone wells were divided into two of the 24 well plates and the supernatant from four of the non-Poly-Lysine coated well plates were divided into two wells of the 24 well plate. The remaining wells were labeled as control and inserted into a Synergy Biotek reader for results.

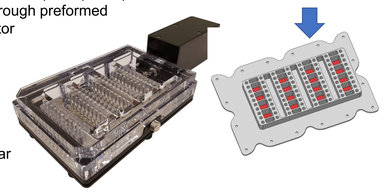
ASC differentiation assays. ASC's were also observed for differentiation when placed in different media after confluency in a T-75 flask. When ASC's were placed in Osteoqual media we observed osteocytes interacting with each other in seven days and could be stained with Alizarin red dye. When ASC's were placed in Adipoqual media we observed adipocytes interacting with each other in seven days and could be stained with Oil red-O dye.

- **Data on the initial optimization of human adipose stem cell (ASC) growth on the stretchable MCFX plates are provided.**

The MechanoCulture FX (MCFX) Machine

The MechanoCulture FX device uses a flexible silicone cell well plate (arrow) which allows for cell culture and visibility. Metal pins go through preformed holes which lock the well in place that moves by an actuator which can pull the silicone well and distort cells adhered to the bottom of the well. The silicone well plates are 8mmx8mm and have 16 available wells to culture cells. Cells can be observed in the silicone wells under microscopy.

The device can be programmed by a user to their particular set of parameters to observe the effects of uniaxial stretch on cells that have adhered to the wells. Once programmed the device can run independently of a computer.



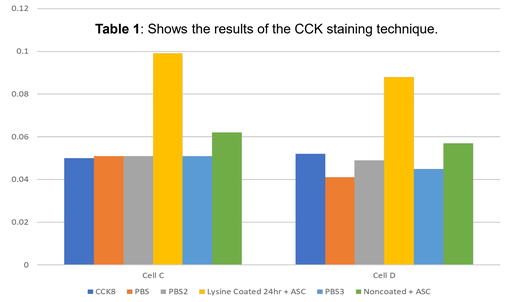
Results

1. Effect of time of Lysine Coating on ASC adhesion and growth on the MCFX culture plates

Results

	1	2	3	4	5	6	
A	0.050	0.051	0.050	0.050	0.052	0.051	450
B	0.050	0.055	0.050	0.051	0.047	0.051	450
C	0.050	0.051	0.051	0.099	0.051	0.062	450
D	0.052	0.041	0.049	0.088	0.045	0.057	450

Table 1: Shows the results of the CCK staining technique.



2. Crystal violet staining enabled the visualization of ASCs on the MCFX culture plates

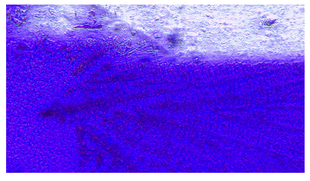


Image 1: Crystal Violet Stain of ASCs grown on plates coated with Poly-L-Lysine for only 4 hours.

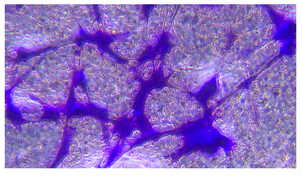


Image 2: Crystal Violet Stain of ASCs grown on plates coated with Poly-L-Lysine for 24 hours.

3. ASCs could be differentiated to adipose and osteocyte lineages on culture plates.

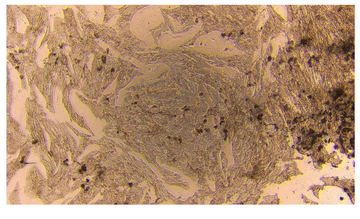


Image 3. Alizarin red staining of ASCs differentiated into Osteocytes for 12 days.

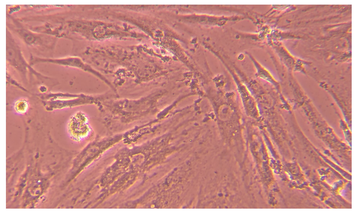


Image 4. Oil red-O staining of ASCs differentiated into Adipocytes for 12 days.

Discussion

Discussion: Creating an *in vitro* model to explore the molecular mechanisms that explain the favorable outcomes of osteopathic manipulative techniques (OMT), particularly the myofascial release (MFR) technique, is a crucial stride towards improving our comprehension of the healing effects of these techniques. This model facilitates the examination of the impact of stretch and release on the function of mesenchymal stem cells (MSC) and their secretome, which could offer a better understanding of the mechanisms behind the advantageous effects of MFR on stem cells that reside in the tissue and how they react to the tissue's microenvironment.

The MechanoCulture FX (MCFX) machine, a programmed stretch and release device, is used to replicate the MFR procedure *in vitro*. This unique method enables controlled manipulation of MSCs to imitate the effects of MFR on tissue-resident stem cells. A critical step in developing this in vitro model is the optimization of procedures for MSC culture on a flexible silicone membrane and their mechanical manipulation utilizing the MCFX device. According to the study's preliminary findings, MSCs can be grown on the silicone membrane, remain attached after being stretched and released, and develop into adipocytes or osteocytes when exposed to static circumstances. The data comparing MSC proliferation and differentiation under stretch and static conditions is currently being collected and will provide further insights into the effects of stretch and release on MSC function.

Limitations: There are some limitations to this work, despite the fact that the creation of an in vitro model of MFR is a crucial step in improving our knowledge of the therapeutic benefits of these methods. First, the complex mechanical and biochemical signals that cells encounter in vivo may not be completely replicated by using a stretched silicone membrane to imitate the tissue microenvironment. Second, using just two MSC varieties could not accurately reflect the variety of MSCs discovered in vivo. To fully comprehend the impact of stretch and release on MSC function and their secretome, future studies utilizing a variety of MSCs from various tissue origins and donors would be required.

Future Studies: The utilization of this in vitro model has the potential to establish a basis for subsequent research that examines how stretch and release impact MSC proliferation, differentiation, and modifications in their secretome. This approach can be broadened to investigate the effects of MFR on other cell types, such as fibroblasts or immune cells, which could contribute to the advantageous effects of OMT. Additionally, using this model to scrutinize the effects of different MFR techniques or varying durations of stretch and release may provide further understanding of the mechanisms that underlie the therapeutic effects of these techniques. Finally, forthcoming studies could also explore the impact of other osteopathic manipulative techniques by using comparable in vitro models, which could offer insights into the mechanisms that generate the beneficial effects of these techniques.

Conclusion: Our *in vitro* model is the first step to examining the effects of MFR on myofascial stem cells. This may provide the foundation for future studies comparing the effects of stretch and release on MSC proliferation, differentiation, and changes in their secretome. Use of this in vitro model may simulate the effects of MFR on tissue-resident stem cells and their responses to the tissue microenvironment and may elucidate how this osteopathic technique enables its well-known beneficial effects *in vivo*

References

1. Bhattacharya S, Wolfenson H, Shivdasani R, Shalom-Feuerstein R. **Stem cell responses to stretch and strain.** Trends Cell Biol. 2022 Jan;32(1):4-7. doi: 10.1016/j.tcb.2021.10.007. Epub 2021 Nov 17. PMID: 34801376.https://pubmed.ncbi.nlm.nih.gov/34801376/

2. Tran RDH, Morris TA, Gonzalez D, Hetta AHSHA, Grosberg A. **Quantitative Evaluation of Cardiac Cell Interactions and Responses to Cyclic Strain.** Cells. 2021 Nov 17;10(11):3199. doi: 10.3390/cells10113199. PMID: 34831422; PMCID: PMC8625419.

3. Abramochkin DV, Lozinsky IT, Kamkin A. **Influence of mechanical stress on fibroblast-myocyte interactions in mammalian heart.** J Mol Cell Cardiol. 2014 May;70:27-36. doi: 10.1016/j.yjmcc.2013.12.020. Epub 2014 Jan 2. PMID: 24389344.

4. CellScale: MechanoCulture FX
1. http://www.shinningtech.com/pdf/MechanoCulture-TR.pdf

Acknowledgements

We like to thank Dr. Mondal for teaching us the proper techniques used to carry out this project. We would also like to thank LMU-DCOM for providing the funding needed for our current studies. The research was supported by a Student Scholar Award grant to Student Doctor Ben Smith (OMS-IV).