Mechanical stimulation *in vitro* regulates pro-inflammatory cytokines: potential insight into soft tissue manual therapies for osteopenia and sarcopenia

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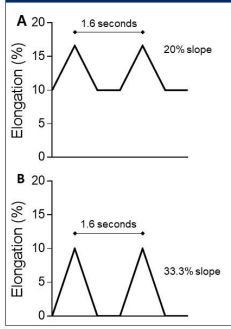
Abstract

Objective: Soft tissue manual therapies are commonly utilized by osteopathic physicians, chiropractors, physical therapists and massage therapists. These techniques are predicated on subjecting tissues to biophysical mechanical stimulation but the cellular and molecular mechanism(s) mediating these effects are poorly understood. Previous studies established an in vitro model system for examining mechanical stimulation of dermal fibroblasts and established that cyclical strain, intended to mimic overuse injury, induces secretion of numerous pro-inflammatory cytokines. Moreover, mechanical strain intended to mimic soft tissue manual therapy reduces strain-induced secretion of pro-inflammatory cytokines. Here, we sought to partially confirm and extend these reports and provide independent corroboration of prior results. We have also begun to extend these reports through the use of differentiation assays using osteoblastic precursors from both calvaria and long bone cells. We observe this differentiation into osteoblasts from the precursors when they are exposed to conditioned media containing the pro-inflammatory cytokines from the aforementioned mechanical strain and therapy studies.

Results: Using cultures of primary human dermal fibroblasts, primary human skeletal myocytes, and murine C2C12 satellite cells, we confirm, in fibroblasts, that cyclical mechanical strain increases levels of IL-6 and adding long-duration stretch, intended to mimic therapeutic soft tissue stimulation, after cyclical strain results in lower IL-6 levels. We also extend the prior work, reporting that long-duration stretch results in lower levels of IL-8 in fibroblasts, as well as provide novel data showing cytokine changes in the myocytes and satellite cells. Although there are important limitations to this experimental model, these findings provide supportive evidence that therapeutic soft tissue stimulation may reduce levels of pro-inflammatory cytokines. In addition, we have exposed MC3T3E1 cells, an osteoblastic precursor from calvarial bone, and murine tibiae to the previously mentioned conditioned media containing these pro-inflammatory cytokines to observe its potential differentiation upon exposure. While this study did not provide any significant findings in the MC3T3E1 line, it gave us key insight into our next steps for the differentiation assays. We also exposed another osteoblastic precursor, W-20-17, to the previously mentioned conditioned media. Gene expression analyses suggest that soft tissue manipulation (STM) increases osteoblast differentiation, as determined by expression of the osteoblast marker Osteocalcin. However, these preliminary results were not statistically significant and will need to be replicated before confirming this preliminary analyses. The tibiae showed an increased expression of the osteoblastic marker, P1NP and a decrease in CTx-1, a marker for bone resorption. One of these insights has led us to begin observing different cell lines, specifically cells originating from long bone. Future work is required to address these open questions and advance the mechanistic understanding of therapeutic soft tissue stimulation.

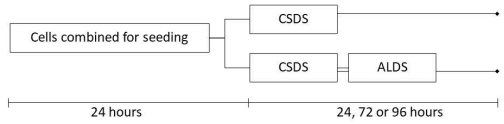
Mechanical Strain Profiles



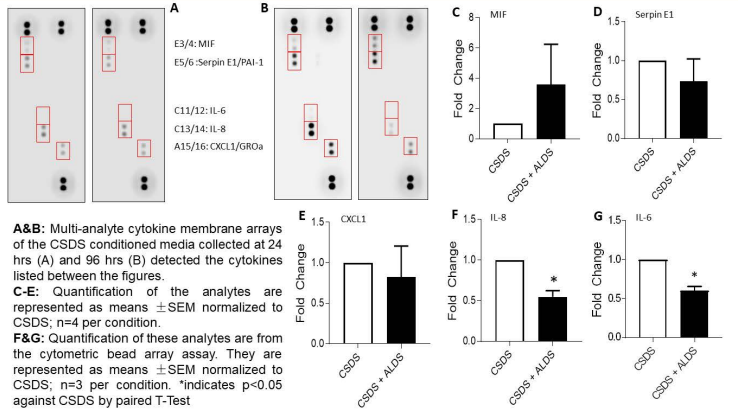
Figures A and B represent a cyclic short duration strain (CSDS) profile that previous researchers () have developed to represent a repetitive motion strain.

Figure C: Human primary dermal fibroblasts, human skeletal muscle myocytes (HSkMC), and mouse C2C12 myoblasts were seeded separately onto flex plates coated with collagen I and subjected to a CSDS profile (A or B) alone, or a CSDS profile (A or B) followed by 3 hours of rest, then an acyclic long duration stimulation (ALDS) profile in which cells were elongated to 6% for 60 seconds with a loading rate of 3%/second and a release at 1.5%/second. Conditioned media was collected at 24 and 96 hours after treatment for fibroblasts, and 72 hours after treatment for C2C12 cells and HSkMCs.

C

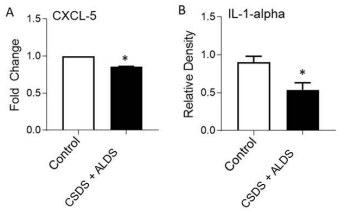


Primary Dermal Fibroblasts



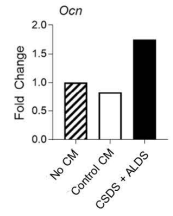
A&B: Multi-analyte cytokine membrane arrays of the CSDS conditioned media collected at 24 hrs (A) and 96 hrs (B) detected the cytokines listed between the figures.
C-E: Quantification of the analytes are represented as means ±SEM normalized to CSDS; n=4 per condition.
F&G: Quantification of these analytes are from the cytometric bead array assay. They are represented as means ±SEM normalized to CSDS; n=3 per condition. *indicates p<0.05 against CSDS by paired T-Test

C2C12 & HSkMCs



A represents pilot data of HSkMC. **B** represents pilot data of C2C12 myoblasts. Quantification is by by multi-analyte membrane array, and is represented by means ±SEM normalized to control; n=2 per condition. *indicates p<0.05 against control by paired T-Test.

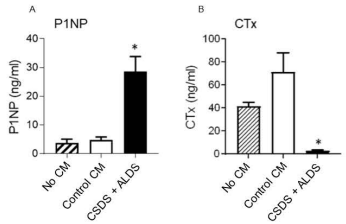
Osteoblastic Precursor Differentiation



qRT-PCR data for osteoblast marker Osteocalcin in W-20-17 cells exposed to osteogenic media +/- conditioned media (CM) from dermal fibroblasts subjected to mechanical strain through CSDS + ALDS

Tibiae Explant Differentiation

Enzyme-Linked Immunosorbent Assay (ELISA) results for bone formation marker P1NP (**A**) and bone resorption marker CTx (**B**) from neonatal tibiae explants exposed to osteogenic media +/- conditioned media (CM) from dermal fibroblasts subjected to cyclic short duration strain (CSDS) followed by acyclic long duration strain (ALDS) or control. * indicates p<0.05 compared to control conditioned media



Conclusions

The ALDS profile was developed to be representative of soft tissue manipulation (STM) techniques such as myofascial release. The fibroblast data and preliminary data for C2C12 myoblasts and HSkMCs show decreased release of pro-inflammatory cytokines, IL-6 and IL-8, in particular, with the ALDS treatment. Chronic IL-6 exposure has been shown to accelerate disease progression and have several effects on skeletal muscle, including decreased muscle protein synthesis and increased protein degradation, which ultimately results in muscle wasting. Reduction of these pathways by STM may reduce the effects of the muscle wasting that occurs in sarcopenia.

Preliminary differentiation assays have allowed us to see the affect of the conditioned media on bone explants and osteoblast precursors themselves. Original data from tibiae explants shows a statistically significant increase in bone formation marker P1NP and a statistically significant decrease in bone resorption marker CTx. Preliminary analysis of an osteoblastic precursor cell line, W-20-17, also suggests that when exposed to STM influenced conditioned media, there is an increase in osteoblast marker Osteocalcin. This preliminary data gives us a promising insight into potential treatments for osteopenia and osteoporosis, as we are observing the potential for bone resorption to be slowed down and bone formation to potentially be increased. Further experimental data is needed before any certainty can be conveyed.

References & Acknowledgements

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