Dosed Myofascial Release in Three-Dimensional Bioengineered Tendons: Effects on Human Fibroblast Hyperplasia, Hypertrophy, and Cytokine Secretion

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Abstract

Objective: The purpose of this study was to investigate potential differences of magnitudes and durations associated with dosed myofascial release (MFR) on human fibroblast proliferation, hypertrophy, and cytokine secretions.

Methods: Bioengineered tendons (BETs) attached to nylon mesh anchors were strained uniaxially using a vacuum pressure designed to model MFR varying in magnitudes (0%, 3%, 6%, 9%, and 12% elongation) and durations (0.5 and 1-5 minutes). Conditioned media were analyzed for cytokine secretion via protein microarray (n = 2). Bioengineered tendons were weighted and fibroblasts extracted from the BET were assessed for total cell protein and proliferation via double-stranded DNA quantification (n = 5). All data were compared by a 1-way analysis of variance with post hoc Dunnett test and Student *t* test.

Results: Changing MFR magnitude and duration did not have an effect on total fibroblast cellular protein or DNA accumulation. However, we observed a stepwise increase in BET weight with higher-magnitude MFR treatments. Longer durations of MFR resulted in progressive increase in the secretions of angiogenin, interleukin (IL)-3, IL-8, growth colony–stimulating factor, and thymus activation–regulated chemokine. Alternatively, increasing strain magnitude induced secretions of IL-1β, monocyte chemoattractant cytokine, and regulated and normal T cell expressed and secreted chemotactic cytokine.

Conclusion: Cellular proliferation and hypertrophy were not significantly changed by any treatment. However, the change in total BET dry weight suggests that production of extracellular matrix protein may be up-regulated. Different MFR parameters induce secretions of a unique subset of cytokines and growth factors that can be further enhanced by increasing the magnitude and duration of treatment. If clinically translatable, these results suggest that variations to manual therapy biomechanical parameters may differentially affect physiological responses in vivo. (J Manipulative Physiol Ther 2013;36:513-521)

Key Indexing Terms: *Musculoskeletal Manipulations; Fascia; Tendons; Fibroblasts*

Myofascial release (MFR) is a specialized form of manual manipulative therapy designed to treat a variety of conditions such as musculoskeletal injuries, somatic pain, fibromyalgia, and chronic lymphedema.[1-4] In some cases, MFR has been shown to improve mobility, reduce pain, and reduce inflammation.[5-7] Treatment typically consists of a slow-loading stretch applied to the long and transverse axis of restricted connective tissue for 90 to 120 seconds, with palpation pressure varying based on anatomy, injury type, and tissue response.[8] During manipulation, the clinician feels changes in tissue texture, thus becoming more palpable. It is believed that manual pressure can alter the plasticity of the connective tissue by modifying its viscoelastic and piezoelectric properties.[8] Despite these reports, there are few studies that provide mechanistic evidence to support these observations. In addition, clinical studies investigating the efficacy of MFR have been inconsistent, with some

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Paper submitted January 8, 2012; in revised form May 31, 2013; accepted July 29, 2013.
0161-4754/$36.00
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http://dx.doi.org/10.1016/j.jmpt.2013.07.004

studies reporting positive clinical outcomes and others showing no difference compared with conventional standard of care.[9-11] Many of these clinical studies focus primarily on a specific manual therapy technique rather than describing the biomechanical parameters used in these maneuvers. This makes it difficult to compare clinical relevance from different studies. For example, one study investigating the effects of dosed cervical mobilization reported that higher frequencies of the same maneuver enhanced sympathetic efferent activity and increased cutaneous blood flow in the upper limb.[12] This suggests that there may be a potential physiological effect of dosed manual manipulative therapy.

Fibroblasts are the primary cell type of connective tissue, that is, fascia, tendons, and ligaments, and provide biomechanical support to the body. Their primary role is to maintain extracellular matrix homeostasis through secretion, degradation, and reorganization of collagens, proteoglycans, fibronectins, tenascins, and laminins.[13] Recent findings suggest that fibroblasts are essential to facilitating the adaptive immune response and wound healing through the secretion of specific cytokine and growth factors such as type 1 interleukins (ILs) and IL-1β, which are involved in leukocyte preservation.[14,15] The multifunctional ability of fibroblasts to respond uniquely to biomechanical strain and their relative abundance within the connective tissue nominate them as a likely candidate of mechanotransduction during manual manipulative treatments.

Using video recordings of clinically applied MFR, we previously measured the strain direction, frequency, duration, and magnitudes applied during the technique and use these parameters to develop an in vitro model of MFR by seeding fibroblasts on Bioflex plates.[16] We have shown that modeled MFR effectively normalizes fibroblast apoptosis, proliferation indices, and actin architecture, as well as simultaneously acting to suppress secretions of various inflammatory cytokines that are induced by a modeled repetitive motion strain (RMS).[16-18]

The purpose of this study is to determine whether variation in modeled MFR strain magnitude or duration can induce unique fibroblast cellular response. This study improves on our previous 2-dimensional in vitro model by using 3-dimensional bioengineered tendons (BETs) to more closely mimic the physical environment found in vivo. In addition, we also investigated the dose-dependent effects of modeled MFR durations and strain magnitudes on fibroblast hyperplasia, hypertrophy, and secretion of cytokines and growth factors. We hypothesized that variation in MFR strain duration and or magnitude will generate unique cellular responses. The results from this study may help to explain clinically why clinicians whom use slightly different maneuvers might obtain different results and provide a proof of concept to establish a basis for the quantification and standardization of MFR and other manual manipulative techniques in the clinical setting.

Methods

Cell Culture

All experiments were conducted using commercially available normal human dermal fibroblasts obtained from Cambrex Laboratories (East Rutherford, New Jersey). The protocol was reviewed by the University of Arizona Office of Human Research Protections and is exempted from institutional review board review. Cells were cultured in Dulbecco modified Eagle medium supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin at 37°C, 5% CO_2, and 100% humidity. The medium was replaced every other day with fresh prewarmed growth medium. Subconfluent cultures (acquired in 7-10 days) were passaged at a ratio of 1:3; all experiments used cell passages between 4 and 10.

Fabrication of BET

Human fibroblasts (1000 cells/μL) were added to a 70% Purecol collagen type I (Advanced Biomatrix, San Diego, CA)–30% 5 × Dulbecco modified Eagle medium mixture to create a collagen/fibroblast gel. A loading trough was created for the collagen/fibroblast gel using a linear Trough Loader placed beneath the flexible membranes of the Tissue Train Plates (Flexcell Int, Hillborough, NC). These plates are arranged in a 6-well format and consist of a flexible elastomeric well bottoms attached to a nondeformable matrix bonded by nylon mesh anchors. A vacuum of −85 kPa was then applied to create a trough between the 2 anchors into which 200 μL of the collagen/fibroblast gel was then added to the fabricated loading trough to create a tube shape structure attached at the 2 anchors. The collagen was allowed to polymerize for 2 hours in a humidified 37°C incubator. Subsequently, the vacuum was released, thereby allowing the BET to be free from attachments to the well, except at the anchor points (Fig 1A). Fresh culture medium supplemented with 2% fetal bovine serum was then added to each well, and the BETs were allowed to acclimate for 24 hours before commencing strain treatment.

Biomechanical Strain Paradigm: In Vitro Dose Response of MFR

All strain profiles were implemented using the Flexercell FX-4000 Tension Plus System (Flexcell International Corp). Vacuum pressure is applied at the elastomeric well bottom, which strains the BET uniaxially in the direction of its primary axis (Fig 1B). In this 3-dimensional model, there are many factors that contribute to overall biomechanics. The uniform structure, elasticity, and ductile nature of the tissue allow the BET to deform under tensile stress. With increasing strain, the length of the BET also increases, which causes the mass to be redistributed across the structure. The phenomenon demonstrated by this process is a Poisson effect, in which a transverse strain is generated perpendicularly to the direction of applied load.[19] This

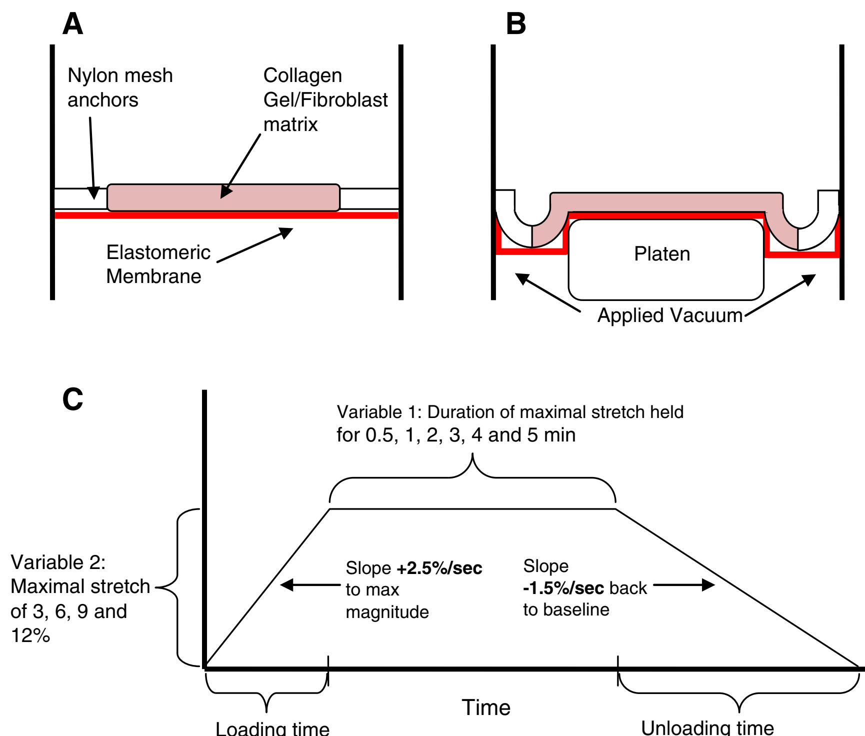

Fig 1. *A, Schematic of the BET attached to nylon mesh anchors at resting position. B, A stationary platen is then placed beneath the well bottom. A controllable vacuum pressure is applied to the system to uniaxially strain the BET in the direction of the long axis of the structure. C, Representation of a slow-loading and unloading MFR strain profile with varying strain magnitudes (3%, 6%, 9%, and 12% elongation) and durations of the static hold (0.5, 1, 2, 3, 4, and 5 minutes). (Color version of figure is available online.)*

effect is the greatest at the midline of the structure, and the change in cross-sectional area is the greatest at this point. The specific parameters (frequency, direction, and loading rates) of the in vitro MFR model were previously determined by analyzing videomorphic data of clinically applied MFR.[16] The modeled MFR strain profile consisted of a slow-loading strain that elongated the tissue beyond its initial resting length at a rate of 2.5%/s until maximum magnitude was reached. This MFR model was modified to incorporate a time- and magnitude-dosed MFR to determine the extent to which these variations cause unique cellular responses in fibroblast hyperplasia, hypertrophy, and cytokine secretions. In the time-dosed MFR treatments, BETs were elongated to 6% beyond their original resting length, at a rate of 2.5%/s, and held for 0, 0.5, 1, 2, 3, 4, or 5 minutes. The strain was then released until baseline length was reached at a rate of −1.5%/s. In the magnitude-dosed treatments, the same strain loading and unloading rates were used; however, BETs were elongated to 0, 3%, 6%, 9%, or 12% beyond its resting length, held for 90 seconds, and returned to baseline (Fig 1C). Bioengineered tendons were sampled 48 hours after treatment, a time point that was chosen based on our previous work showing a minimum of 24 hour for changes in cell proliferation, apoptosis, and secretion of inflammatory cytokine and growth factors to be detectable.[16,17]

Proliferation, Intracellular Protein, and Dry Mass Measurements

The total number of fibroblasts contained within each BET was insufficient to accurately measure cell proliferation via cell count. Therefore, double-stranded DNA (dsDNA) quantification method was used because it offered greater sensitivity for proliferation measurements. To analyze indices of fibroblast proliferation and hypertrophy, fibroblasts were extracted from the BETs 48 hours after strain. Fibroblasts were harvested from BETs using 0.2% collagenase (Sigma Aldrich, St Louis, MO) digestion at 37°C with gentle mixing for 1 hour. The cell solution was filtered through a 40-μm nylon cell strainer and centrifuged at 2000g for 15 minutes. The supernatant was removed, and the cells were washed twice in phosphate buffer saline. The samples were recentrifuged and resuspended in 100 μL of cell lysis buffer containing protease inhibitors (Roche Diagnostics, Indianapolis, IN). The samples were then sonicated under low setting using a microsonicator and assessed for growth measurements and cellular protein expression. Cell proliferation was measured as a proxy of quantified total cell

Table 1. *BETs strained by modeled MFR at 6% elongation for 0.5-to 5-minute and 90-second MFR at 3%, 6%, 9%, and 12% maximum elongation*

	Modeled MFR						
	NS	0.5 min	1 min	2 min	3 min	4 min	5 min
DNA (pg/mL)	26.77 ± 0.6	27.3 ± 0.3	26.3 ± 0.1	26.6 ± 0.2	26.4 ± 0.4	26.2 ± 0.3	27.1 ± 1.4
Protein (μg/mL)	223 ± 20.4	187.6 ± 16.6	251 ± 28.9	186.8 ± 14.3	166.5 ± 28.8	183.6 ± 18.1	193.7 ± 15.7

	NS	3%	6%	9%	12%
DNA (pg/mL)	190.8 ± 2.5	193.9 ± 1.2	196.1 ± 1.4	198.5 ± 0.7	200.0 ± 2.0
Protein (μg/mL)	522 ± 16.5	507.4 ± 27.1	537.7 ± 16.6	535.7 ± 9.9	528.1 ± 15.6

Total dsDNA and intracellular protein were measured 48 hours after cessation of strain, and data are expressed as mean ± SEM.
NS, non-strain; *MFR*, myofascial release.

dsDNA using the CellTiter 96Aqueous One Solution cell proliferation assay according to the manufacturer protocol (Promega Corp, Madison, WI). Total protein expression was determined using the BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL). For the duration- and magnitude-dose response experiments, 5 independently cultured BETs were collected to determine total fibroblast DNA content and total protein expression. To determine total mass of the constructs, BETs were physically detached from the anchors using microforceps and placed in preweighed 250-μL Eppendorf tubes. Bioengineered tendons were then desiccated in a 37°C hybridization chamber for 24 hours to remove aqueous solution. Five independent BETs from duration- and magnitude-dosed treatments were weighed using an analytical scale. Bioengineered tendon masses were determined by subtracting the total weight from the preweighed Eppendorf tubes.

Cytokine Quantifications

A controlled regulated inflammatory response is vital in the early phases of tissue injury and repair. Secreted cytokines and growth factors stimulate cell migration and proliferation within the injury site. In this study, we screened for 42 different soluble cytokines and growth factors involved with angiogenesis, inflammation, and chemotaxis by analyzing BET-conditioned media with commercially available cytokine antibody arrays (RayBiotech, Inc, Norcross, GA). Glass slides (arranged in an 8-sample format) coated with antibodies were incubated with blocking buffer, followed by conditioned media from each treatment group in duplicate. Slides were then incubated with a detection biotin-conjugated cytokine antibody cocktail (Alexa Fluor 532–conjugated streptavidin; Raybio-tech, Inc, Norcross, GA) and fluorescent dye conjugated with streptavidin. Signals were detected with an Agilent Scanner G2505B and analyzed for pixel intensity via GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA); data were further quantified with Q-Analyzer v3.5 (RayBiotech, Inc). Three separate conditioned media samples were tested for each treatment group. All data were corrected for culture media volumes before statistical analyses.

Statistical Analysis

Data are shown as mean ± SEM for all groups and were compared by Student *t* test and a 1-way analysis of variance (ANOVA) with post hoc Dunnett tests using Prism 4.03 (GraphPad Software, Inc, San Diego, CA). Group means with $P < .05$ determined by ANOVA were verified by *t* tests and were considered significantly different.

Results

Fibroblast Proliferation and Hypertrophy Are Unaffected by Varying Durations and Magnitudes of Strain

After 48 hours poststrain, we did not observe any significant changes in total BET DNA content in any group tested. Similarly, total cellular protein did not significantly change among any of the treatment groups (Table 1).

Modeled MFR Increases Total BET Weight in a Dose-Dependent Manner

Different durations of MFR treatment did not significantly affect total BET weight (Fig 2A). However, increasing the magnitude of the MFR paradigm from 3% to 12% lead to a stepwise increase in total BET dry mass (Fig 2B). The 12% strain group resulted in the greatest increase in dry weight of 76% when compared with the nonstrain control (Fig 3).

MFR Induces Secretion of Cytokines and Growth Factors in a Dose-Dependent Manner

We investigated 42 different strain-induced fibroblast-derived cytokines that promote inflammation and wound healing. Varying the MFR magnitudes led to significant changes in 3 of the 42 cytokines tested. Increasing MFR strain magnitude led to stepwise increases in the secretion of IL-1β, regulated and normal T cell expressed and secreted chemotactic cytokine (RANTES), and monocyte chemoattractant cytokine (I-309) when compared with the nonstrain control. Secretion of RANTES and I-309 was significantly increased in the 12% MFR treatment group, and a significant increase of IL-1β was observed in 9% MFR groups when compared with control. Holding the strain magnitude

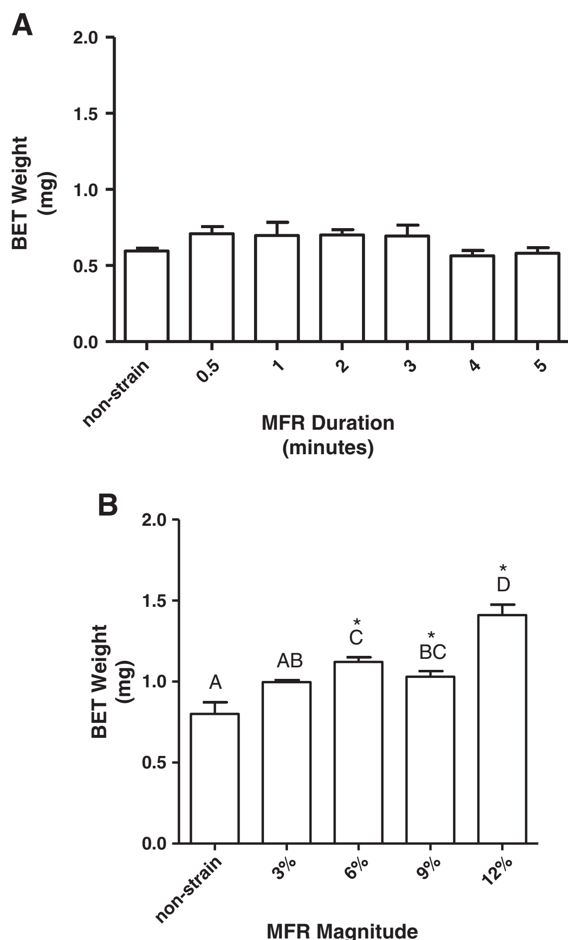

Fig 2. *Total dry weight of BET 48 hours after treatment with time (A; 0.5, 1, 2, 3, 4, and 5 minutes) and magnitude-dose (B; 3%, 6%, 9%, and 12% elongation) MFR. Different letters represent P < .05 (t test) comparing respective groups. *P < .05 (Dunnett 1-way ANOVA) vs control. BET, bioengineered tendons; MFR, myofascial release.*

constant at 6% and varying the duration of the treatment resulted in an observed increase in the secretion of growth-regulated oncogene, IL-3 and IL-8, growth colony–stimulating factor (GCSF), angiogenin, and thymus activation–regulated chemokine. Significant increases of these cytokines were only observed in the 5-minute MFR treatment groups ($P < .05$) when compared with control. These data are summarized in Figure 3 and Figure 4.

Discussion

One of the issues associated with clinical studies evaluating manual therapies efficacy is the degree of variability in techniques of both intraclinician and interclinician reproducibility.[20-24] The complexity of such studies and the difficulty associated with objective quantification methods to measure positive outcomes are dependent on multiple subjective factors including the patient's mood, pain tolerance, and perception of pain. As a result, there is a wide range of conflicting findings such as those reporting that manual therapy intervention improves strength, reduces pain, and improves function in patients with osteoarthritis and neck and shoulder pain,[1-4,25] whereas other findings show no differences from the conventional standard of care.[26-28] We have previously used in vitro models to reproducibly and objectively study the effects of biomechanical strain modeling MFR on fibroblast physiology. In this study, we aimed to improve our model by using in vitro BETs to investigate the cellular effects of variations of MFR magnitudes and durations. We observed that different MFR strain profiles were capable of inducing differential responses in cytokine and growth factor secretions as well as changes in overall mass of the BET structure. To our knowledge, this is the first study to investigate dose responses of modeled MFR in vitro. These findings suggest and highlight the importance of the standardization of techniques to obtain consistent results or desired outcomes.

With regard to mechanical stress, fibroblasts have been shown to be both force sensors and effectors of strain. Fibroblasts respond to mechanical forces by inducing molecular and cellular changes specific to the biomechanical environment.[29,30] We have previously shown in 2-dimentional constructs that treatment with a 60-second 6% MFR applied equibiaxially is insufficient to induce a proliferative or hypertrophic response in human fibroblasts.[16] In this current study, we observed similar results using a 3-dimensional tissue construct. Neither increasing strain magnitudes (3%, 6%, 9%, 12%) nor increasing strain durations of the holding period (0.5, 1, 2, 3, 4, and 5 min) had a significant impact on total DNA or fibroblast protein content (Table 1). However, we have previously reported that MFR-applied heterobiaxial strain resulted in a significant increase in fibroblast proliferative and hypertrophic responses.[18] The differing in mitogenic responses resulting from different static strain mechanics suggests that fibroblast proliferation may be a strain direction and/or tissue structure (3-dimension vs 2 dimension)–related phenomenon.

We also observed an increase in total BET dry weight in higher-magnitude MFR treatment groups. The increase in tissue weight with no observed change in DNA content or total cell protein suggests that the increase in BET mass is likely caused by an increase in extracellular matrix production. It is reported that fibroblast secretion of extracellular matrix protein is regulated by mechanical strain.[31,32] Determining the correlation of our strain model with increased collagen secretion is currently an area under investigation.

Inflammation is an essential physiological response to tissue injury and repair. However, unregulated and chronic

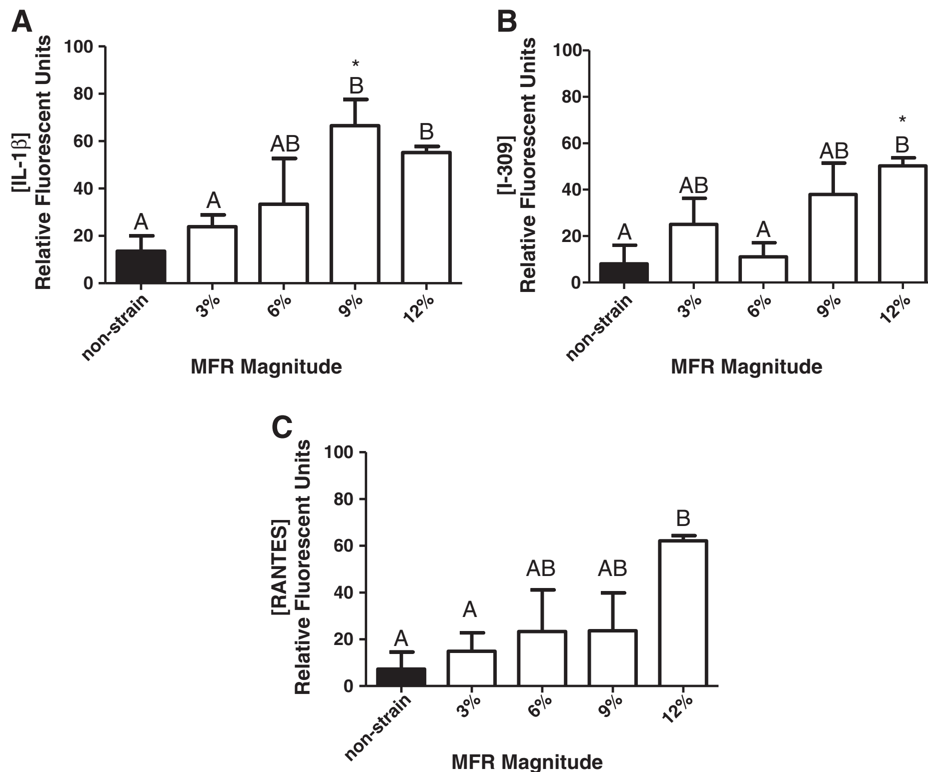

Fig 3. *Bioengineered tendons strained with a modeled MFR for 90 seconds at 3%, 6%, 9%, and 12% maximal elongation. Of the 42 cytokines measured in the conditioned media 48 hours poststrain, only IL-1β (A), chemokine I-309 (B), and RANTES (C) were found to be significantly affected by strain. Values measured as a mean relative fluorescent unit (n = 2-3). Different letters denote P < .05 (Student t test). *P < .05 (Dunnett ANOVA analysis) vs nonstrain control. MFR, myofascial release.*

inflammation may result in inappropriate tissue damage leading to rheumatic diseases.[33] Manual therapies have been shown to be effective treatment for alleviating systemic and localized acute inflammation.[34,35] However, the mechanism that manual therapy regulates inflammation remains elusive. New evidence is beginning to support new roles for fibroblast in the immune-inflammatory response. Activated fibroblast in response to injury can lead to rapid secretion of inflammatory cytokines and growth/chemotactic factors that regulate hematopoietic cells activity. Fibroblasts then self-inactivate once homeostasis is restored. In diseased conditions such as rheumatoid arthritis, synovial fibroblasts extracted from these joints lose the ability to self-inactivate and constitutively secrete inflammatory cytokines.[36] We have previously shown that modeled MFR alone is insufficient to induce cytokine secretions[16,17] in 2-dimensional fibroblast cultures. However, when MFR was applied after RMS, it effectively suppressed the secretion of proinflammatory cytokines (ie, IL-1α, IL-1β, IL-2, IL-3, and IL-6) induced by RMS,[17] suggesting that MFR is capable of inactivating the fibroblast inflammatory phenotype. Furthermore, we have shown that MFR-regulated changes to cytokine secretions may alter the phenotype of adjacent cells such as the underlying skeletal muscle.[37] In our 3-dimensional tissue constructs, modeled MFR resulted in no change to cytokine secretions when using similar biophysical parameters to the 2-dimensional model. However, increasing strain magnitude and duration resulted in specific stepwise increases of both inflammatory cytokines and growth factors (Figs 4 and 5). These results suggest that by varying the parameters of the biomechanical stimuli, MFR can serve as both an activator and an inhibitor of fibroblast-mediated inflammation. In vivo, fibroblasts express specific genes and cytokine secretion profiles that depend on their anatomical location,[38,39] a characteristic that is maintained in in vitro cultures. It was also shown that altering the local inflammatory environment can alter the transcriptional profile of the localized fibroblast population.[40] Our results indicate that biomechanical strain also modifies fibroblast gene expression, which is dependent on the duration and magnitude of strain, a phenomenon that was not evident in 2-dimensional constructs at the time and magnitude tested. Preliminary studies from our laboratory suggest that stretch-activated calcium channels may play an important proximal step in strain-mediated gene expression by coding for

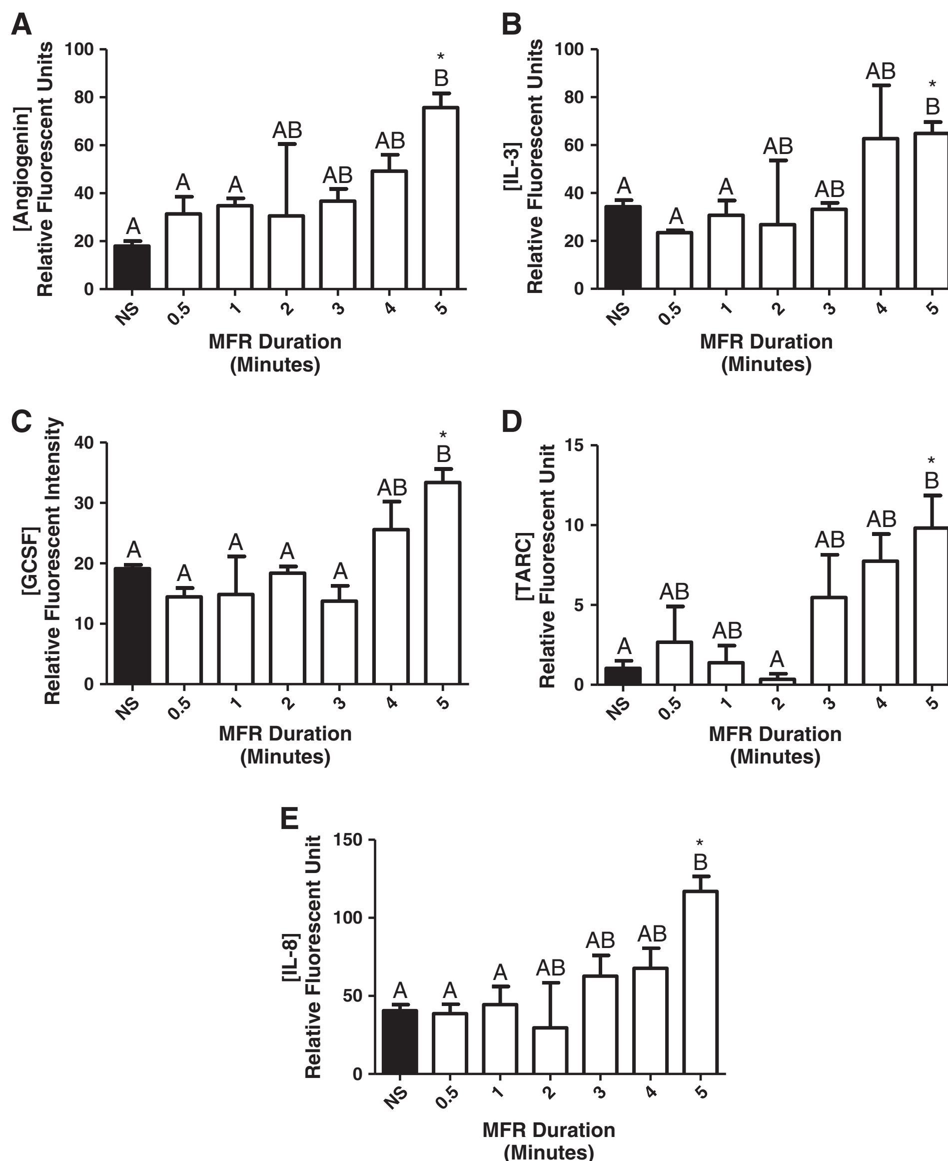

Fig 4. *Bioengineered tendons strained with a single dose of modeled MFR at 6% maximal elongation for 0.5-, 1-, 2-, 3-, 4-, or 5-minute durations. Cytokines were measured in the conditioned media by protein microarray 48 hours poststrain. Of the 42 cytokines measured, only angiogenin (A), IL-3 (B), GCSF (C), thymus activation–regulated chemokine (D), and IL-8 (E) showed significant changes in response to strain. Data reported as mean relative fluorescent units (n = 2-3). Different letters denoted P < .05 (Student t test) compared with respective groups. *P < .05 (Dunnett ANOVA analysis) vs NS, nonstrain control. MFR, myofascial release.*

intracellular calcium pulses that result in gene activation and vesicle release. We are currently investigating the stretch-activated calcium channel as a potential mechanism for fibroblast strain-induced cytokine secretion.

Limitations

This 3-dimensional model allows us to more closely mimic the in situ environment and provide vast improvements over our 2-dimensional studies. However, it is also important to note that limitations still exist in correlating these data with clinical outcomes. With this methodology, we cannot reproduce many of the aspects that are produce clinically such as pressure, changes in tissue temperature upon contact and friction, and stimulation of sensory nerves. We did not factor nor consider the other cell types and organ systems that are affected by the clinician during manipulation such as underlying muscle, blood vessels,

nociceptors, and so on, which may also contribute to the clinical outcomes. This is beyond the scope of our study because we are only interested in the effects of the biomechanics that are sensed by the fibroblasts. With this in vitro model, we can apply a consistent and reproducible mechanical load to quantify fibroblast responses. From these data, we identify possibilities for which manual therapy may mechanistically induce graded physiological effects.

Conclusion

The study results suggest that variations in biomechanical parameters translate to a diverse array of responses and potential clinical outcomes. Interestingly, up-regulation of cytokine secretions is highly dependent on both strain magnitude and duration. If these data are clinically translatable, these results implicate efficacy for both prophylactic and dose-dependent MFR that may potentially regulate inflammation and wound healing responses. The in vitro model models presented in this study may prove advantageous and useful in refining the techniques and application of manual therapy in the clinical setting.

Practical Applications

- Fibroblasts generate unique cellular response to different parameters of biomechanical strain.
- Longer-duration and higher-magnitude MFR up-regulates a distinct subset of inflammatory mediators and appears to be dose dependent.
- Changes in the artificial tendon dry weight with no observed change in fibroblast hyperplasia and hypertrophy also suggest a potential regulation of extracellular matrix production.

Funding Sources and Potential Conflicts of Interest

Funding for this study was provided by the American Osteopathic Association (Grant No. 10-21-623) No conflicts of interest were reported for this study.

Contributorship

Concept development (provided idea for the research): TVC, MRH, DC, PRS.
Design (planned the methods to generate the results): TVC, MRH, DC, PRS.
Supervision (provided oversight, responsible for organization and implementation, writing of the manuscript): TVC, MRH, DC, PRS.
Data collection/processing (responsible for experiments, patient management, organization, or reporting data): TVC, MRH, DC, PRS.
Analysis/interpretation (responsible for statistical analysis, evaluation, and presentation of the results): TVC, MRH, DC, PRS.
Literature search (performed the literature search): TVC, MRH, DC, PRS.
Writing (responsible for writing a substantive part of the manuscript): TVC, MRH, DC, PRS.
Critical review (revised manuscript for intellectual content, this does not relate to spelling and grammar checking): TVC, MRH, DC, PRS.

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