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Effect of Collagen Digestion on the Passive Elastic Properties of Diaphragm Muscle in Rat

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Abstract

Effects of collagen digestion have been defined up to the fibril level. However, the question remains as to whether the alteration of skeletal muscle extracellular matrix (ECM) affects a muscle's passive elastic response. Various elastography methods have been applied as tools for evaluating the mechanical properties and ECM content of skeletal muscle. In an effort to develop an ECM altered skeletal muscle model, this study determined the effect of collagen digestion on the passive elastic properties of skeletal muscle. Passive mechanical properties of rat diaphragms were evaluated in various degrees of collagen digestion. Between cyclic loading tests, muscle strips were immersed in various concentrations of clostridium histolyticum derived bacterial collagenase. All samples were later viewed via light microscopy. Cyclic testing revealed linear relationships between passive muscle stiffness and digestion time at multiple concentrations. These results demonstrate that collagenase digestion of the ECM in skeletal muscle could be used as a simple and reliable model of mechanically altered *in vitro* tissue samples.

Keywords

Extracellular matrix; biomechanics; myopathy; elastography

Introduction

The extracellular matrix (ECM) of muscle acts to support blood vessels and nerves, and is a major source of passive resistance to stretch[1]. The ECM is indirectly connected to the contractile proteins of the muscle cell through the subsarcolemmal cytoskeleton[2]. This relationship along with previous work[3-5] makes it likely that the ECM is involved in lateral force transmission within the muscle and critical in the transmission of external stimuli to the muscle cells. Increased levels of matrix metalloproteinases that act to breakdown the ECM have been reported in cases of hyperthyroid myopathy[6] and idiopathic inflammatory myopathies[7]. Given the potential importance of the ECM in force transmission and

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mechanotransduction, the increased breakdown of the ECM may contribute to the symptoms of the myopathies.

Monitoring the passive stiffness of a muscle seems to be a logical way to indirectly assess the breakdown of the ECM. Techniques such as magnetic resonance elastography[8] and ultrasound elastography[9, 10] offer the possibility of doing this in vivo. In fact, magnetic resonance elastography has been used to show changes in myopathic muscle[11, 12]. While it seems intuitive that the digestion of the ECM would result in reduced stiffness of the muscle, this has never been tested on the whole muscle level. Prado and colleagues[1] demonstrated that the ECM plays a role in the passive stiffness of a muscle by removing the titin. However, as some of the contributions of titin would be passed through the ECM when examining whole muscle stiffness, the effects of removing the ECM are likely to be larger than the contribution attributed to the ECM by Prado and colleagues. A review by Huijing highlighted the need for further study of collagenous components found in endomysium. The purpose of the present study aims to determine the effect of collagen digestion at the muscle level and provide an in vitro model of collagen digestion for assessment of ECM breakdown.

Material and Methods

The adult wistar rats (n=4) were euthanized with carbon dioxide and the diaphragm muscle was rapidly removed. Two 5 mm-wide muscle strips were extracted from the midcostal region of each hemidiaphragm. To prevent the degradation, the diaphragm strips were immediately immersed in a modified Rees-Simpson saline solution, which was aerated with 95% O_2-5% CO_2 and maintained at 26°C [13] within 15 minutes of sacrifice. The Institutional Animal Care and Use Committee at Mayo Clinic approved all experimental procedures.

Mechanical testing

Passive stiffness of each sample was evaluated using a custom built mechanical tester (Figure 1). On one side of the tester, samples were clamped to a load cell (Transducer Techniques, Temecula, CA) with a resolution of 1 mN. On the other side, samples were clamped to a linear servo-motor (Parker MX80L) driven displacement stage. Displacement was controlled by a custom LabVIEW program. Displacement was recorded by a LVDT (Novotechnik, Germany) attached to the stage. Throughout the testing process, samples were suspended horizontally in a bath containing the previously mentioned Rees-Simpson saline solution, to prevent dehydration.

Slack length of the muscle strips was defined as the length at which the muscle strips experienced 5 mN of tensile force. After determining their slack lengths, the samples were submitted to cyclic loading. Initially, samples were preloaded up to 120 % of their slack length. Once preloaded, samples were cycled at a rate of 1 mm/s and an amplitude of 20% of their slack length (Figure 2). Samples were submitted to 10 pre-conditioning load cycles. Afterwards, samples were allowed to rest for five minutes prior to the actual cyclic testing. During each subsequent test, samples were submitted to 5 loading cycles.

All samples were submitted to 4 progressive rounds of cyclic testing with 5 minute breaks between each round. Each round contained 5 cyclic loading/unloading tests with the displacement amplitude and displacement rate the same as the preconditioning cycles. During the five minute break, 4 muscle samples were soaked in solutions containing 0.25%, 0.125%, 0.0625%, and 0% type II clostridium histolyticum, respectively, derived bacterial collagenase (GIBCO, Invitrogen Corporation), in Rees-Simpson solution. As tensile strain is known to greatly reduce skeletal muscle's susceptibility to enzymatic digestion, samples were left in a relaxed state during digestion periods[14].

For each sample, passive stiffness was determined from the slope of the linear portion of the fifth hysteresis curve generated by each cyclic test (Figure 3). The linear portion was defined as the region between the peak force and 25% of the peak force. Slope of the linear region was calculated by means of a linear regression. The samples of the same collagenase concentration were averaged. To normalize the stiffness, each data set was multiplied by the scaling factor necessary to make its maximum data point equal to 1.

One-way-ANOVA test was performed on stiffness values of the control specimens and the specimens digested with various collagenase concentrations at the end of 15 minutes. Tukey's post-hoc test was performed to identify the pair(s) with statistically significant difference. In addition, at each collagenase concentration, linear regression was performed between the normalized stiffness and the digestion time.

Histology

As a means to verify collagen changes, light microscopy was used to determine the histological effects of the collagen digestion process. Following the mechanical measurement, each sample was fixed in 10% formalin. Once sufficiently bound, samples were imbedded in paraffin. Five 4μm thick longitudinal slices were taken from each muscle strip. Slices were fixed to slides and stained using a standard Masson Tri-chrome technique. Slides were imaged at 40x, 100x, and 400x magnification. Collagen networks were identified as the blue stained structures surrounding the red stained muscle fibers. Degree of collagen degradation was assessed on a qualitative basis by the same observer.

Results

Mechanical testing

Regression analysis revealed strong linear relationship (r^2=0.97 for 0.0625% concentration; r^2=0.98 for 0.125% concentration; r^2=0.99 for 0.25% concentration) between normalized stiffness and digestion time at all concentrations (Figure 4). Samples digested in all the three collagenase concentrations exhibited statistically significant reduction in normalized stiffness at the end of the tests (p<0.01) when compared to the control samples. In addition, the difference in normalized stiffness reduction between samples treated with 0.0625% collagenase and those treated with 0.125% collagenase was statistically insignificant (p=0.5). The differences in normalized stiffness reduction between samples treated with 0.125% collagenase and those treated with 0.025% collagenase was also statistically insignificant (p=0.11). (Figure 4)

Histology

Figure 5 shows the ECM degradation after digestion in all collagenase concentrations. ECM morphology remains normal in the control muscle sample (Figure 5 row 1). Samples digested in 0.0625% collagenase (Figure 5 row 2) exhibited no significant change in the intensity of the epimysium. In contrast, samples digested in 0.125% collagenase (Figure 5 row3) exhibited an obvious disassociation in the epimysium, and a slightly less pronounced disassociation in the interior of the muscle. In samples digested with 0.25% collagenase (Figure 5 row 4), the epimysial layer of the ECM was disappeared. Further degradation was also noticed in the interior of the 0.25% collagenase digested samples.

Discussion

Cyclic testing performed in various degrees of collagen digestion revealed a clear linear relationship between digestion time and passive stiffness in diaphragm muscle. These results verified that collagenase has a predictable effect on both the mechanical and histological properties of skeletal muscle. Although previous study[15] produced similar results in engineered

collagen matrices, this study revealed how the extracellular matrix organization of skeletal muscle responds to collagen digestion. As the collagenase used in this study was introduced to the muscle samples by means of immersion and infiltration, the most pronounced effects of the digestion were found in the outermost portions of the muscle. Although light microscopy revealed epimysial collagen dispersion in all digested samples, collagen reduction on the endomysial and perimysial levels were only prominent in samples digested in high (0.25%) collagenase concentration.

Strong collagen networks in the endomysium and perimysium of skeletal muscle are one of the essential components for the distribution and transmission of tensile force. As has been previously discussed in the literature, intracellular proteins and structures also contribute to passive stiffness and ultimately the transmission of forces to tendon[16]. Therefore, tensile loads applied to samples digested in 0.25% collagenase would likely be transferred through fewer pathways leading to the reduced stiffness seen in this study. Suki & Bates[17] have thoroughly demonstrated that interconnections in a tissue's ECM are essential for the tissue to maintain its stiffness. Consequently, it is reasonable that the samples digested in 0.25% collagenase exhibited a larger reduction in stiffness than those digested in lower concentrations. In the same way, the samples digested in the two lower concentrations were similar because neither group underwent collagen digestion on the perimysial or endomysial levels.

The major fibrillar collagens in epimysium, perimysium and endomysium are type I and type III collagens, while the major fibrillar collagens in basement membranes are types IV collagen[18]. The collagenase used in this study has been shown to be quite specific in its digestion[19]. In addition it has been used as a standard method to isolate cells with minimal damage[20]. Therefore, we believe that the muscle fiber will remain grossly unaffected after digestion. As only samples digested in the 0.25% collagenase solution showed substantial changes in the endomysium and perimysium, the concentration seems to be appropriate for future use in testing the ability of methodologies that can be applied in vivo to assess collagen digestion. As the stiffness continued to decrease with additional digestion time up to 15 minutes, altering digestion time seems to be a logical way to establish different levels of digestion with this model.

This study had three notable limitations. First, all mechanical tests in this study were preformed on rat diaphragm muscles. The architecture of the muscle strips tested is significantly simpler than that of most muscles. Second, the digestion was achieved by immersion and infiltration. While this is substantially different from the in vivo digestion that would occur in myopathies, the current methodology did achieve digestion at all ECM levels when using the highest collagenase concentration. Third, this study did not attempt to quantify the amount of collagen lost from digestion. Such information would assist in clarifying the specific relationship between collagen content and passive muscle stiffness. The results of the current study do present important initial framework for further research.

Conclusion

The current study demonstrated that collagen digestion has substantial effects on muscle stiffness at the tissue level. The study also demonstrated that samples digested in a 0.25% collagenase solution show substantial changes in the endomysium and perimysium, indicating this concentration seems appropriate for future use as an in vitro model of collagen digestion.

Acknowledgments

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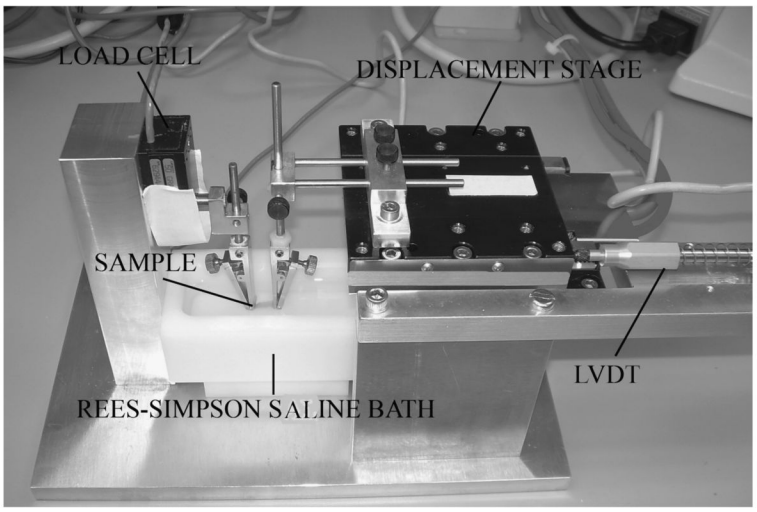

Figure 1.
In vitro mechanical testing apparatus.

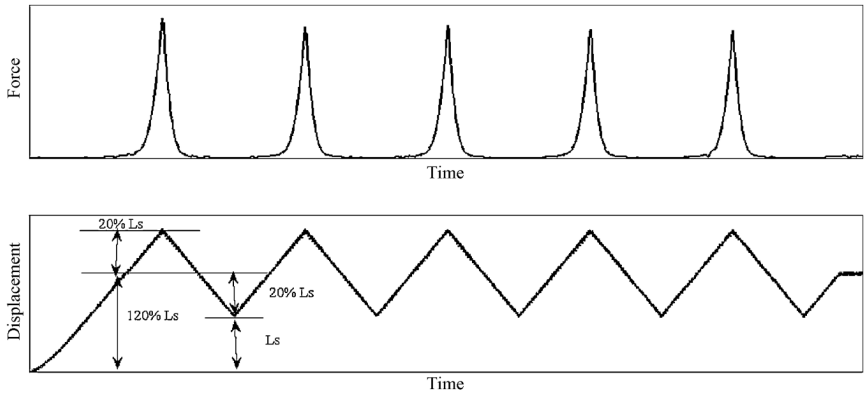

Figure 2.

The cyclic loading process. The force versus time plot (top), shows the five loading cycles. A sample profile of the displacement of the tendon versus time is seen in the bottom figure. Samples were preloaded to 120% slack length (L_s) and cycled at amplitude that equals 20% slack length.

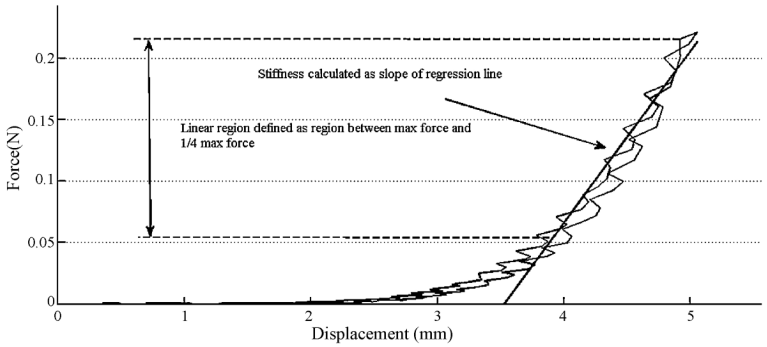

Figure 3.

An example of the force versus displacement curve, as obtained during mechanical testing. Stiffness was calculated using the hysteresis curve corresponding to the fifth cycle of each round of cyclic tests. Stiffness was calculated as the slope in the linear region.

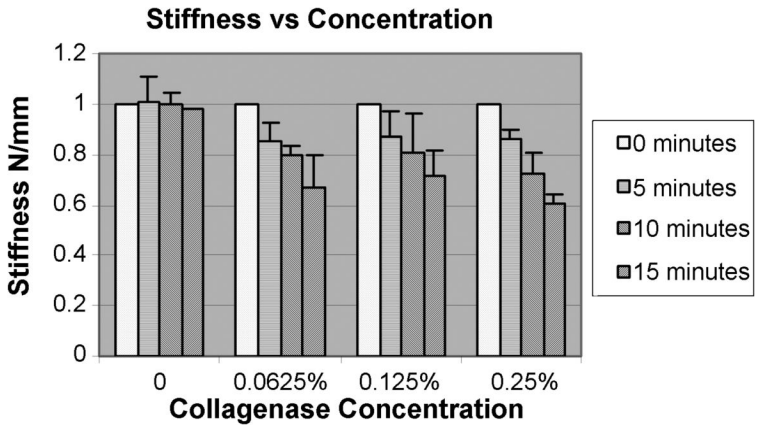

Figure 4.
Normalized stiffness vs. collagenase concentration (0.0625%, 0.125%, 0.25%) at various digestion duration time (0, 5, 10, and 15minutes).

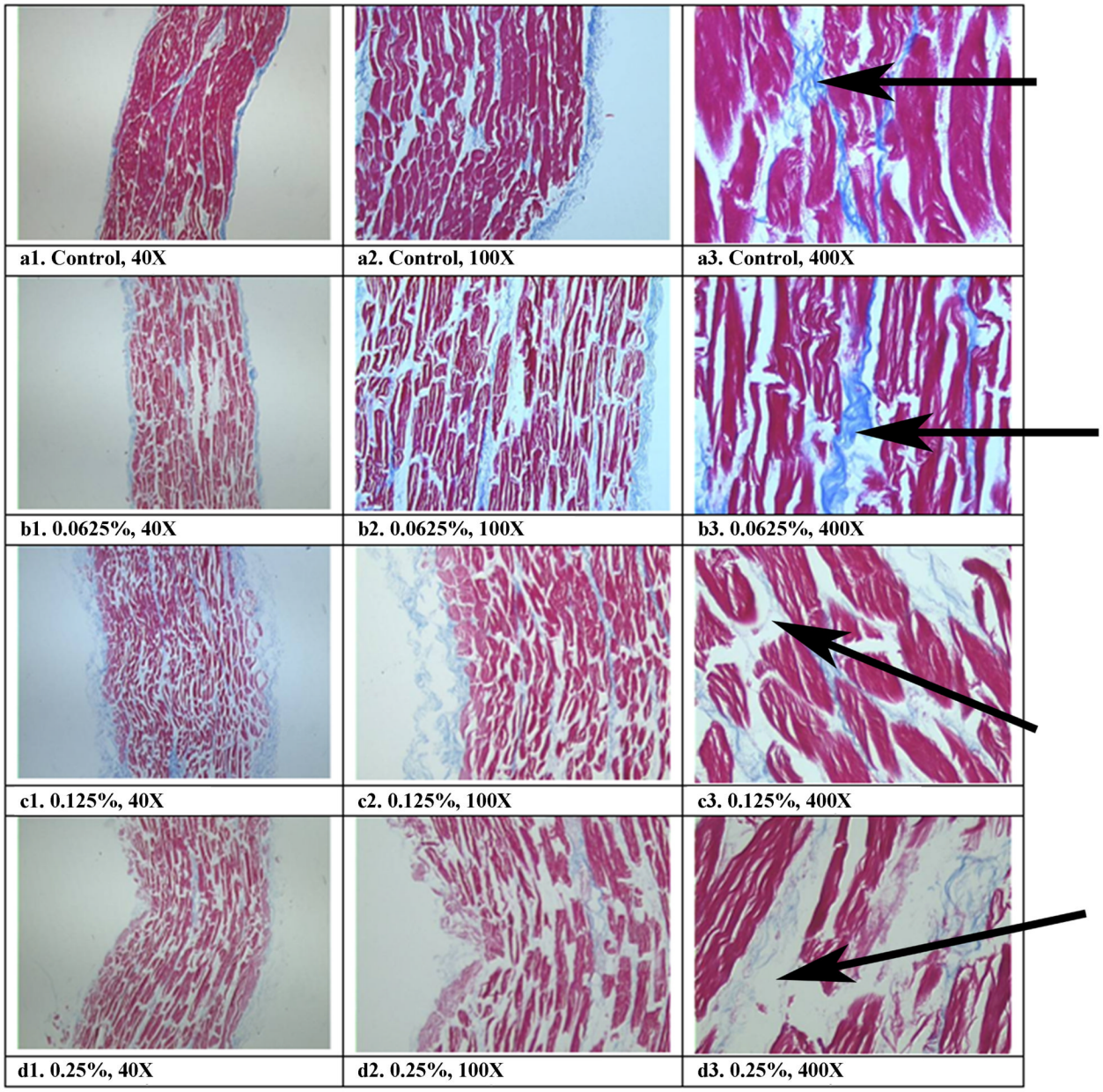

Figure 5.
Histology images of the muscle specimens. Slides were stained using the Masson trichrome technique. Collagen can be identified by the black arrows and appears as stained tissue within the red stained muscle fibers. Considerable amounts of collagen were still visible at the 0.0625% concentration, however 0.125% and 0.25% show a considerable decrease in stained collagen between fibers, indicating substantial digestion.