PLASMA AND SKELETAL MUSCLE MATRIX METALLOPROTEINASE-9 RESPONSE TO ECCENTRIC EXERCISE-INDUCED INJURY IN HUMANS

by

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Exercise induced muscle injury is a phenomenon that most adults have experienced when beginning a new or novel exercise program. It is perceived as muscle soreness developing 24-48 hours after completion of the exercise task. The role of the muscle extracellular matrix (ECM) in remodeling of muscle during the repair process following injury has recently become a focus of this area of research. Key to the remodeling of the ECM is the inducible protease, matrix metalloproteinase-9 (MMP-9) that preferentially degrades type IV collagen of the ECM. Animal models have provided support for MMP-9 in this remodeling process but the role of MMP-9 following exercise induced muscle injury in the human model remains controversial.

The purpose of this dissertation was to provide a more definitive examination of the plasma and skeletal muscle MMP-9 responses following exercise induced muscle injury in humans. To accomplish this goal, three models were used: elbow flexion, downhill running, and leg extension. Rigorous controls related to subject inclusion/exclusion criteria, exercise protocols, and methods for measurements, were incorporated into each of the studies to address confounds in previous work. Unlike previous studies, a test-retest measure was added to provide information on the stability of the dependent measures from day-to-day.

We consistently found a significant increase in plasma MMP-9 immediately after eccentric exercise, but the magnitude was small and rarely exceeded the variation measured from day-to-day. We concluded that plasma MMP-9 is not an adequate marker of muscle damage, but
this does not preclude a role at the level of the tissue. Inside muscle, we found signs of ECM modification following eccentric actions, supporting the idea that eccentric actions stimulate ECM remodeling. The early timing of the plasma MMP-9 response, along with the presence of ECM changes within muscle, is consistent with one of the proposed mechanistic roles for MMP-9, degradation of ECM to assist with the migration and invasion of immune cells. Future work that focuses on changes inside of muscle with increased sampling time points and includes measurement of immune cells is needed to clarify the specific role of MMP-9 in exercise induced muscle injury in humans.
DEDICATION

To my Mother:

In losing you, I gained a passion to understand physiology.
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GENERAL INTRODUCTION

Exercise-induced muscle soreness is a feeling that most people have experienced at some point in life. This muscle discomfort is distinct from performance fatigability (Kluger et al., 2013) because the onset of the soreness is delayed by 1-2 days following completion of the exercise task. Delayed onset muscle soreness (DOMS) has been reported to contribute to diminished performance in the occupational (Cutlip et al., 2009) and athletic arenas (Smith, 1992). It has been shown to cause impairment in activities of daily living (Clarkson & Hubal, 2002), especially in the most susceptible populations (e.g., sedentary individuals and older adults (Ploutz-Snyder et al., 1998)). Published studies examining the physical changes associated with DOMS have been documented for over 100 years (Hough, 1900), though there is no known cure or prevention strategy. A historical report dated prior to the first published account of the DOMS phenomenon suggests that the “supportive environment that surrounds muscle” is perhaps as important in physiological processes as muscle (Bowman, 1840). However, it wasn’t until more recent years that muscle damage and repair processes have been expanded to include the network of connective tissue proteins blanketing skeletal muscle, known as the extracellular matrix (ECM). The purpose of this general introduction is to provide an overview of the structure and function of the ECM and discuss the potential role of the ECM in skeletal muscle damage and remodeling processes in humans with a particular emphasis on one member of a family of extracellular matrix degrading proteases, matrix metalloproteinase-9. The introduction will also establish the rationale for a series of studies presented in this dissertation.
The structure and function of the extracellular matrix surrounding skeletal muscle

The ECM is a medium through which forces are transmitted and is composed of collagenous and non-collagenous proteins that function to provide support, strength, and elasticity to skeletal muscle (Kovanen, 2002; Kjaer, 2004; Grounds et al., 2005). Skeletal muscle is surrounded by ECM macromolecules at each level of organization including whole muscle (epimysium [EP]), muscle fascicles (perimysium [P]), and muscle fibers (endomysium [E]) (Figure 0-1).

*Figure 0-1.* Electron micrograph of skeletal muscle extracellular matrix after muscle has been digested away from the ECM. The epimysium (EP) is shown in the upper left image, perimysium (P) and endomysium (E) are labeled in the bottom image, and the top right image shows the endomysium around a single muscle fiber (Adapted from Kjaer, 2004).
The endomysium, the area of ECM that is in closest proximity to the sarcolemma, is composed of two laminar layers (basal and reticular) (Sanes, 2003) that are collectively referred to as the basement membrane (Figure 0-2). In addition, in the interstitial space between muscle fibers there are also interstitial connective tissue proteins that form connections with the basement membranes of adjacent muscle cells.

![Figure 0-2. Electron micrograph image defining the layers of the extracellular matrix relative to the cell membrane and sarcomeres (Adapted from Grounds et al., 2005).](image)

The primary constituents of the basal lamina are type IV collagen and a non-collagenous family of protein, laminins (mostly isoforms 2 and 4) (Kovanen, 2002; Grounds et al., 2005). Type IV collagen and laminin self-assemble to form a covalently-bound structural network that is linked to the more superficial reticular layer by non-collagenous adhesive glycoproteins (entactin, nidogen, perlecan) (Sanes, 2003)(Figure 0-3). The covalent bonding of collagen and laminin allows for organizational flexibility with assembly and disassembly of the lattice network of ECM macromolecules in response to changes in the nearby cellular and interstitial environment (Alberts et al., 2002). The basal lamina is linked to the sarcolemma below through
transmembrane receptor proteins, dystroglycan and integrin (Han et al., 2009), which are thought to be important sensors in the mechanotransduction of forces that initiate cellular signaling pathways in muscle (Kjaer, 2004; Larsen et al., 2006). Inside of the muscle fiber, the cytoplasmic protein, dystrophin, is responsible for linking dystroglycan complexes to the actin cytoskeleton.

**Figure 0-3.** Schematic of the general molecular structure of the basal lamina, primarily composed of type IV collagen, laminin, entactin, and perlecain (Adapted from http://illuminationstudios.com/archives/139/molecular-structure-of-basal-lamina).

Together with linkages to cytoskeletal structures, integrin and dystrophin proteins allow the ECM and muscle to move in a coordinated fashion in response to perturbations (Komulainen et al., 1998; Lovering & De Deyne, 2004; Koh & Escobedo, 2004). Without all of the intricate linkages of the ECM to motor proteins of sarcomeres, it would not be possible to effectively
transmit the force from contraction that is required to move and stabilize the body (Grounds et al., 2005). This structural foundation of the ECM is rich in content and diversity of macromolecules. While the ECM was once thought to serve as a mere scaffolding for the cells it housed within, it is now apparent that this spider web of proteins that shrouds all muscle cells is influential to a variety of muscle functions and adaptations (Figure 0-4).

Figure 0-4. Electron micrograph image of collagen fibrils of the extracellular matrix (Adapted from Kim & Mooney, 1998).

Beyond providing structural support to muscle, the ECM is also a medium through which mechanical signals are transduced to muscle, initiating a number of intracellular signaling pathways (e.g., ERK1/2, MAPK, Akt, NF-κB) that are sensitive to mechanical signals (Kjaer, 2004; Gillies & Lieber, 2011; Calve & Simon, 2012). There is also evidence to suggest that
proteins of the ECM may either directly (as ligands) or indirectly (through liberation of matrix-bound ligands) stimulate muscle receptors to initiate transcriptional regulation of muscle cells (Kovanen, 2002; Sanes, 2003; Kjaer, 2004; Adair-Kirk & Senior, 2008). Fragments of ECM derived from elastin, connective tissue glycoproteins, or collagen that were proteolytically cleaved during ECM modification have been termed “matrikines” (Maquart et al., 2004). Matrikines have been proposed to be mediators of a number of damage and repair processes, including inflammation (Adair-Kirk & Senior, 2008), chemotaxis (Senior et al., 1989), cell proliferation, and satellite cell and neutrophil migration (Henson & Vandivier, 2006). Indirectly, the ECM may also be responsible for initiating muscle-signaling pathways when growth factors (transforming growth factor-β [TGF-β], fibroblast growth factors [FGFs], vascular endothelial growth factors [VEGFs]) are liberated from the web of ECM proteins as it is remodeled (Kovanen, 2002; Hynes, 2009), allowing for growth factor binding to muscle receptors and downstream transcriptional changes in muscle. As the role of the ECM in muscle cell signaling has become increasingly apparent, attention has been shifted to understanding more about the molecules that are capable of modifying the ECM, and because of their ability to degrade the majority of ECM proteins (Sternlicht & Werb, 2001), matrix metalloproteineases (MMPs) are the leading contenders of interest.

*Matrix metalloproteinases*

The assembly of macromolecules that make up the ECM are specific to the tissue that they surround (Vracko & Benditt, 1972). Since type IV collagen is one of the primary constituents of the basal lamina that surrounds skeletal muscle tissue, molecules that are capable of modification of type IV collagen are potentially important research targets in situations when remodeling of the ECM is likely. While studying the metamorphosis of tadpoles, a group of
investigators discovered a family of proteolytic enzymes capable of degrading the triple helical collagen surrounding skeletal muscle (Gross & Lapiere, 1962). These enzymes were named matrix metalloproteinases (MMPs) because of their dependence upon metal ions for their activation (Brinckerhoff & Matrisian, 2002). Currently, 24 MMPs have been identified in humans (Fanjul-Fernández et al., 2010; Klein & Bischoff, 2011). The MMPs that are the most biologically active in skeletal muscle are MMP-2 (Gelatinase A, 72 kDa type IV collagenase) and MMP-9 (Gelatinase B, 92 kDa type IV collagenase) (Koskinen et al., 2002) because their primary substrate is the main constituent of skeletal muscle ECM, collagen type IV (Sternlicht & Werb, 2001). MMP-2 and MMP-9, also called gelatinases, are transcribed as prepropeptides that have an N-terminal signal sequence (“pre” domain) that when removed directs synthesis toward the endoplasmic reticulum to produce soluble secreted MMP proteins (Sternlicht & Werb, 2001) (Figure 0-5). Following the pre domain, there is a “pro” domain that keeps the secreted MMPs latent until removed to expose the catalytic domain. The catalytic domain contains three cysteine-rich repeats and has a zinc binding active site cleft that is specific to certain substrates such as collagen type IV. Collagen type IV has been identified as the main substrate for the gelatinases, but other ECM proteins have been acknowledged as gelatinase substrates as well (Sternlicht & Werb, 2001; Murphy & Nagase, 2008). Finally, there is a hemopexin domain that is connected to the zinc-binding site through a hinge region. The hemopexin domain is thought to influence the binding of tissue inhibitors of metalloproteinases (TIMPs) and the binding affinity of certain substrates (Sternlicht & Werb, 2001).
Figure 0-5. General domain structure of the gelatinases (MMP-2 and MMP-9) (Adapted from Sternlicht & Werb, 2001).

A number of types of cells associated with skeletal muscle tissue have been suggested as the source of gelatinase secretion, including, but not limited to: neutrophils (Li et al., 1999; Faurschou & Borregaard, 2003; Ardi et al., 2007; Kluger et al., 2013), macrophages (Goetzl et al., 1996; Dreier et al., 2001), lymphocytes (Trocme et al., 1998), muscle fibroblasts (Wilhelm et al., 1989), muscle satellite cells (Guérin & Holland, 1995; Balcerzak et al., 2001), myoblasts (Nishimura et al., 2008), and mature muscle fibers themselves (Lewis et al., 2000). Only MMP-2 appears to be secreted constitutively, while MMP-9 displays inducible expression, indicating that it is regulated at the level of transcription as needed (Sternlicht & Werb, 2001; Klein & Bischoff, 2011). For example, primary mouse satellite cells in culture can be induced to express MMP-9 upon treatment with basic fibroblast growth factor (bFGF) or tumor necrosis factor-α (TNF-α) (Allen et al., 2003). MMP-9 expression is particularly inducible during tissue remodeling states (Kherif et al., 1999; Choi & Dalakas, 2000; Koskinen et al., 2001b; Rucavado et al., 2002; Rullman et al., 2007; Zimowska et al., 2008; Rullman et al., 2009). MMPs are also highly regulated post-translationally by inhibitors called tissue inhibitors of metalloproteinases (TIMPs) and it is important not to ignore the role that these might have in the circulating or tissue environment (Nagase & Woessner, 1999; Sternlicht & Werb, 2001). There are multiple levels of regulation of MMP activity including but not limited to: transcriptional activation and repression, post-transcriptional mRNA processing, mRNA degradation, regulated secretion, proteolytic
activation and inactivation, and protein inhibition (Sternlicht & Werb, 2001). Since MMPs have the potential to breakdown the primary constituents of the ECM, there is a fine balance between too much and too little MMP activation, and regulation at all of these levels is essential to tissue homeostasis. In several conditions that are associated with increased muscle damage, such as rhabdomyolysis, polymyositis, dermatomyositis, and Becker dystrophy, circulating levels of MMP-2 or MMP-9 (or both) are elevated, indicating a chronic state of increased matrix degradation (Koskinen et al., 2001a). Conversely, the consequence of insufficient MMP-9 activity is also problematic for tissue homeostasis and is highlighted by a few of the phenotypic characteristics of MMP-9-null mice including: decreased fertility (possibly as a result of altered endometrium remodeling preventing implantation) (Dubois et al., 2000), defective skin wound healing (Kyriakides et al., 2009), delayed bone ossification during development (Vu & Werb, 2000), and reduced angiogenesis (Hanahan et al., 2000). Because MMP-9 is an inducible gelatinase and might be upregulated in response to ECM remodeling, the remainder of this dissertation will be limited to a focus on the role of MMP-9 in muscle damage and repair.

Potential role of MMP-9 in skeletal muscle damage and repair

A variety of models can be used in the laboratory setting to induce muscle injury in animals or humans, each with its advantages and disadvantages (reviewed in (Tiidus, 2008)). In this dissertation, I will be discussing the unique events associated with exercise-induced muscle injury. In the immediate hours to days following an initiating injury event, electron micrograph images of muscle show evidence of physical disruption of sarcomeres in the form of sarcomere misalignment (Newham et al., 1983; Lauritzen et al., 2009), z-disk streaming (Yu et al., 2004), and widened I-bands (Clarkson & Hubal, 2002) (Figure 0-6).
In the subsequent days, a cascade of events to facilitate removal of the damaged cytoskeletal structures ensues (Proske & Morgan, 2001). From about 48-72 hours after the injury, there is histological evidence showing phagocytic cells migrating from the systemic circulation toward damaged fibers via diapedesis for the removal of disrupted myofibrils, cytosolic organelles, and the damaged muscle fiber plasma membrane (Armstrong et al., 1983; Jones et al., 1986; Malm et al., 2000) (Figure 0-7). This phagocytic cellular invasion has been termed the “secondary injury” phase of muscle injury (Faulkner et al., 1993) because some evidence suggests that the infiltrating cells may actually contribute to additional muscle fiber injury (Pizza et al., 2001; McLoughlin et al., 2003).
There is evidence to suggest that chemoattractants produced by injured muscle tissue, such as interleukin-1β (IL-1β), interleukin-8 (IL-8), TNF-α, lipid derivatives (leukotriene B4), or ECM proteins (fibronectin, laminin), might be the signal that attracts neutrophils and monocytes to the location of injury (Grounds & Davies, 1996; Nagaraju, 2001; Tsivitse et al., 2003) and MMP-9 degradation of the ECM may orchestrate this process. The timeline of the phagocytic cell invasion also happens to temporally correspond to the time when human subjects rate the peak of their perceived soreness (Clarkson & Hubal, 2002). In the weeks after a recoverable muscle injury event, quiescent satellite cells that reside outside of the muscle fiber plasma membrane but underneath the basal lamina go through activation, proliferation, and migration to replace damaged fibers by fusing together with themselves or with remaining fibers (Guérin & Holland,
In tissue, histological signs of satellite cell activation (Crameri et al., 2004) and central nucleation (Tidball & Wehling-Henricks, 2007) are present in the weeks after muscle injury, indicative of muscle regeneration (Figure 0-8).

Figure 0-8. Immunohistochemical evidence of a regenerating muscle cell (A) and an activated satellite cell (B) marked by the presence of CD56 in human skeletal muscle following eccentric cycling exercise (Adapted from Malm et al., 2000).

The roles that have been proposed for MMP-9 in the skeletal muscle damage and repair events are generally associated with movement of molecules into or out of the matrix. Specifically, these processes may include degradation of ECM components to potentially assist with the migration and invasion of immune cells to the injured muscle fibers (Sternlicht & Werb, 2001; Carmeli et al., 2004; Klein & Bischoff, 2011), liberation of necessary growth factors from the web of ECM that assist myogenic precursor cells in activation, proliferation, and differentiation (Sternlicht & Werb, 2001; Chen & Li, 2009), and liberation and migration of satellite cells during regeneration. ECM breakdown may be fundamental to each of these processes, and some propose that the orchestrated and timely activity of MMP-9 is crucial to successful tissue repair (reviewed in (Sternlicht & Werb, 2001; Carmeli et al., 2004; Chen & Li, 2009)). Inhibition of MMP-9 by TIMP-1 is associated with processes that prevent degradation of the ECM. When cells are transfected to over-express TIMP-1 and then injected into mice, there
is a reduced metastasis of experimentally-induced tumors (Khokha, 1994). Conversely, increased activity of MMP-9 is clinically associated with processes that favor degradation of the ECM, including increased tumor metastasis (Chambers & Matrisian, 1997; Foda & Zucker, 2001; Deryugina & Quigley, 2006) and diseases characterized by muscle inflammation and weakness (e.g., polymyositis, dermatomyositis) (Koskinen et al., 2001a; Kieseier et al., 2001). These findings suggest that tissue remodeling, in a variety of tissues, is altered when activity of MMP-9 is either reduced or increased. MMPs have been described as the “gatekeeper” of the ECM (Barkan et al., 2010) and MMP-9 in particular may serve as an important therapeutic target for treatment of muscle diseases that depend upon migration of molecules through the dense connective tissue. Currently, there is some information regarding what specific stimuli are capable of initiating an MMP-9 response in cultured cells (Allen et al., 2003; Nishimura et al., 2008; Morgan et al., 2010), and in the circulation or the ECM surrounding animal muscle (Koskinen et al., 2001b; Zimowska et al., 2008; Mehan, 2013), but findings do not always translate to the human model and there is less known about the MMP-9 response to muscle injury, especially when injury is induced by exercise.

Our lab has taken a translational approach toward identifying the role of MMP-9 in the remodeling of skeletal muscle and the ECM using a variety of muscle injury paradigms in both animal and human models. Data from our animal findings supports a role for MMP-9 in muscle damage paradigms in mice. Specifically, our findings from WT mice suggest that circulating MMP-9 is increased after a single bout of downhill running in untrained mice (Mehan, 2013). This finding had also already been reported in at least one human study using a similar downhill running model, but, interestingly, only in subjects who performed the exercise in a cold room (Koskinen et al., 2001a). As other human studies started to report increases in circulating MMP-
following various forms of exercise, we became increasingly interested in determining if we
could translate our lab’s mouse MMP-9 findings to an equivalent human model while controlling
for some of the confounds that we identified in previous human work. In addition, we became
interested in the idea that circulating levels of MMP-9 have the diagnostic potential to serve as a
unique blood marker for the magnitude of muscle damage that has occurred in humans. The
MMP-9 response to exercise models used in humans that is currently published is not consistent
and may be related to the use of a variety of models including exercise tasks that have been
associated with muscle damage as well as tasks that are not. Inclusion and exclusion criteria in
the studies that exist is also inconsistent, which may influence the widely varied magnitude of
muscle damage reported across the different protocols. Since there are a number of issues
regarding the selection of a muscle damaging protocol to use in humans, we decided to look
across three different exercise models that are the most traditionally used in this research field in
humans: eccentric elbow flexion, downhill running, and eccentric leg extension (Figure 0-9). In
each of our experiments, we made a point to control for several variables that may have been
confounds in previous work. Specifically, we were careful to exclude subjects with a recent
history (<6 months) of performing physical activity or similar with the legs. We also excluded
subjects who had recently (<6 months) experienced muscle soreness in the exercising limb(s) to
avoid including subjects who might be protected from damage through the repeated bout effect
(Byrnes et al., 1985). In addition, for all of our studies we gave subjects strict instructions to
avoid physical activity or treatment of any potential soreness with medications or other
therapeutics. Finally, in our first study, we made sure to include an additional pre-exercise time
point so that we could assess the day-to-day variability in the dependent measures that had not
been reported previously.
Figure 0-9 summarizes the series of experiments that we conducted. For our initial experiment, we had subjects perform a single bout of high-force eccentric contractions of the elbow flexors executed over the entire joint range of motion. The elbow flexors are a muscle group that is least likely to be protected from damage through the repeated bout effect from activities of daily living (e.g., walking) (Madden et al., 2011). We put particular emphasis on ensuring that our exercise apparatus was designed to engage the muscle throughout the whole range of motion, as this has been identified as an important variable influencing the magnitude of muscle damage (Nosaka & Sakamoto, 2001). In addition, we were extremely selective with our subject screening process and we only included sedentary subjects without any history of exercise or similar physical or occupational activity for a period of at least 6 months prior to enrollment in the study. Subject enrollment was limited to males to eliminate the potential effect that menstrual hormonal changes might have on the dependent measures in the study (Tiidus, 2000; Clarkson & Hubal, 2001), and our sample size was larger than most of the previously published studies. Finally, we added a repeated pre-exercise visit to assess the stability of the dependent measures in the study and the reliability of our measurement tools, something that had not yet been done by other investigators in this area of research.

In the elbow flexor experiment, we were also interested in a new suggested approach to quantification of changes in muscle force production following eccentric exercise. Specifically, we wanted to examine changes in the muscle force – joint angle relationship and the shift in the optimal joint angle of muscle force production following eccentric contractions that has been documented by several investigators in multiple muscle groups (McHugh & Tetro, 2003; Philippou et al., 2004a; Butterfield & Herzog, 2005; Chen et al., 2007). The presence of an angle shift is based on the concept that stress-susceptible sarcomeres “pop” following eccentric
contractions causing an increase in series compliance in the muscle, and that there may be a
linear relationship between the number of damaged fibers and the length change of the muscle
following damage (Proske & Morgan, 2001). Some have suggested that the angle shift might
serve as a unique index of muscle injury that is more related to the magnitude of sarcomere
damage than is the loss of force following lengthening contractions (Proske & Morgan, 2001;
Warren et al., 2001; Philippou et al., 2004b; Butterfield & Herzog, 2005; Chen et al., 2007), but
very little is known about the reliability of force-angle measurement methods in humans after
muscle injury. Although the data for the force-angle manuscript was collected within the same
elbow flexor protocol used to assess changes in plasma MMP-9 (Dissertation Chapter 2), the
results were worthy of a separate publication and are presented as a separate chapter here
(Dissertation Chapter 1).

After we determined that there was no change in plasma MMP-9 following eccentric
exercise of the elbow flexors in our rigorously controlled and carefully designed initial
experiment, we decided to try the same exercise model that had originally produced the plasma
MMP-9 increase in WT mice in our lab. For the next study, we continued to implement a strict
subject screening process and we maintained several of the previous study design controls, but
we used a downhill treadmill running model (-10°) as the mode of eccentrically-biased exercise.
In addition, we decided to include a subject group of individuals with a history of concentrically-
biased exercise training (e.g., cycling), as there was some suggestion in the literature that
concentrically-biased training is associated with increased susceptibility to lengthening-induced
damage (Ploutz-Snyder et al., 1998; Whitehead et al., 1998; Gleeson et al., 2003). The downhill
running model was more similar to the model we had used for mice in our lab, and it also
involved a larger total muscle mass and repetitive, cyclical contractions, both of which could contribute to an alteration in the detectable plasma MMP-9 response.

For our last study, our main goal was to look at changes in human muscle tissue following eccentric exercise, as the modest and variable plasma MMP-9 changes we had detected in previous experiments raised the question about what was happening at the level of the tissue. Although we detected a small plasma MMP-9 response in subjects following our downhill running protocol, downhill running was the least attractive model to use for a muscle biopsy study since subjects experience damage and soreness in a variety of muscle locations (Byrnes et al., 1985). To maximize our chances of seeing any histological signs of damage, we needed to use a model involving an isolated muscle group. Interestingly, models involving the elbow flexors tend to produce larger CK responses and more strength loss (about 13% greater) (Clarkson & Hubal, 2002), but the elbow flexors pose more risk and difficulty for biopsy sampling than do the knee extensors. Knee extension models of eccentric exercise-induced injury are commonly used in humans and we had the support staff and resources at the Clinical Translational Research Center (CTRC) to perform such an experiment. We were able to address a unique MMP-9 research question in our study by randomizing subjects to exercise groups and having them perform either concentric-only or eccentric-only contractions. We also used a single-leg exercise task so that a biopsy sample from the unexercised leg could serve as each subject’s control specimen for analysis of the results. The role of contraction type has been examined for changes in traditional markers of muscle damage, to some degree, but not for changes in MMP-9 and other proteins associated with remodeling of the ECM. There are very few studies showing changes in both circulating and muscle levels of MMP-9 and other ECM remodeling markers in humans following eccentric or concentric exercise, and the leg extensor
approach is one that could be translated back to a rodent model in the future to examine additional mechanistic research questions in this area.

**Figure 0-9.** Schematic of exercise models used for our lab’s human experiments. Top left: eccentric elbow flexor task; Top right: downhill running task; Bottom left: apparatus used for both the eccentric and concentric (control condition) knee extensor task on weight machine; Bottom right: apparatus used for the eccentric knee extensor task on the isokinetic dynamometer (Original image).

**Scientific goals of thesis**

The scientific goals of this dissertation were 4-fold: 1) To quantify the reliability and measurement error of force-joint angle measurement methods in humans prior to damaging eccentric exercise, evaluate changes in joint angle-muscle force polynomial curves following eccentric exercise and examine the relationship of these changes to traditional markers of muscle damage; 2) To determine the effects of a well-characterized, highly damaging eccentric arm task
on systemic levels and activity of MMP-9 and TIMP-1 in humans while rigorously controlling for the confounding factors such as prior activity level associated with this experimental model; 3) To examine changes in systemic MMP-9 and TIMP-1 levels following downhill treadmill running in two subject populations with a potentially increased susceptibility to lengthening-induced muscle damage; and 4) To examine changes in systemic MMP-9 and TIMP-1, and muscle MMP-9, TIMP-1, and collagen IV mRNA and protein in response to concentric or eccentric muscle actions of the leg extensor muscles in humans. The results in this dissertation clearly advance our body of knowledge for each of these areas as evidenced by inclusion of some of our work in a recent systematic review of the human MMP-9 exercise-induced muscle damage literature (da Cunha Nascimento et al., 2014), along with other references to our published findings in the human skeletal muscle MMP-9 literature that continues to flourish (Silveira et al., 2012; Reihmane et al., 2012; Rullman et al., 2012; Reihmane et al., 2013). Understanding more about the role of MMP-9 in muscle and ECM remodeling in humans could provide valuable insight into clinical conditions characterized by muscle or ECM damage, and could be beneficial to identifying how to tailor exercise-based rehabilitation to facilitate ECM adaptations following muscle injury.
CHAPTER I

REVISITING THE FORCE-JOINT ANGLE RELATIONSHIP FOLLOWING ECCENTRIC EXERCISE

ABSTRACT

The purpose of this study was to evaluate force-angle curve fitting techniques pre-eccentric exercise, quantify changes in curve characteristics post-exercise, and examine the relationship between curve changes and markers of muscle damage. Fourteen males unaccustomed to eccentric exercise performed 60 eccentric contractions of the elbow flexors. Maximal voluntary isometric force was measured throughout a range of angles pre- (Pre1 & Pre2), immediately-post (IP), and 1-, 2-, 4-, and 7-days post-exercise. Force-angle curves for each visit were constructed using 2nd order polynomials. Changes in curve characteristics (optimal angle, peak force, curve height), range of motion, soreness, and creatine kinase activity were quantified. Optimal joint angle and force at optimal angle were significantly correlated from Pre1 to Pre2 (r=0.821 and 0.979, respectively). Optimal angle was significantly right shifted (P=0.035) by 10.4° ±12.9° from Pre2 to IP, and was restored by 1-day post. Interestingly, the r² value for curve fit was significantly decreased (p<0.001) from Pre2 (r²=0.896) to IP (r²=0.802) and 1-day post (r²=0.750). Curve height was significantly decreased (39%) IP and restored to pre-exercise height by 4-days post-exercise. There was no correlation between optimal angle or curve height and other damage markers. In conclusion, force-angle relationships can be accurately described using 2nd order polynomials. Following eccentric exercise, the force-angle curve is flattened and shifted (downward and rightward), but these changes are not correlated to other markers of muscle damage. Changes in the force-angle relationship are multifaceted but determining the physiological significance of these changes requires further investigation.
INTRODUCTION

It has been well established that the performance of a single bout of novel eccentric contractions results in reduction of the force-generating capacity of contracting muscles (Edwards et al., 1977; Warren et al., 1999). The decrease in muscle function associated with this phenomenon has been proposed to have functional significance in the occupational (clinical) and athletic (applied) settings. The loss in muscle force following eccentric exercise has been proposed as an estimate of the muscle damage resulting from such exercise as well as an assessment of recovery from that damage. To be used in the clinical/applied settings to assess muscle damage and recovery, force loss measurements must be reliable and factors contributing to its variability minimized. Warren et. al. have proposed that using force loss as a correlate of physical damage to muscle fibers is consistent with national initiatives to move towards the management of ‘functional outcomes’, but also necessitates uniformity in the measurement approach employed in a rehabilitation setting (Warren et al., 1999).

To assess force loss in humans, many studies have used isometric force measured at a fixed angle before and after an eccentric task. The angle selected is usually the optimal angle for force generation prior to the performance of the eccentric exercise task. However, a number of investigators have reported that there is a shift in the optimal joint angle for peak force production such that peak muscle force output occurs at longer muscle lengths following eccentric exercise (McHugh & Tetro, 2003; Philippou et al., 2004; Chen et al., 2007; Brown & Donnelly, 2011). This phenomenon is most often attributed to an increase in series compliance in the damaged muscle that is thought to occur as a result of disruption to the force-generating element, the sarcomere (Edwards et al., 1977; Warren et al., 1999; Proske & Morgan, 2001; Chen et al., 2007) but may also be the result of fatigue (Warren et al., 1999; Butterfield &
Herzog, 2005). The reported downward and rightward shift in this relationship would result in an overestimation of muscle force loss using the fixed angle method. The variability between individuals in this overestimation of force loss would make it more difficult to use force loss as an estimate of muscle damage and to establish correlations between the magnitude of force loss following eccentric exercise and other parameters that are altered following eccentric exercise. If there is a downward and rightward shift in the force-angle relationship after injury, it is possible that a fixed angle measurement approach used before and after a muscle injury in a clinical/applied setting could contribute to a misjudgment in the magnitude of the injury based on the magnitude of force loss, potentially resulting in a delay in the decision to allow an athlete to return to sport or an employee to return to work after an injury.

By measuring the force produced across a range of joint angles rather than a fixed angle, a more complete picture of the force angle relationship should be obtained and a more accurate estimate of the force loss following eccentric exercise could be determined. In order for force loss to provide an accurate metric of injury as a functional measurement tool in the rehabilitation setting, a new approach may be for a clinician to measure muscle force at multiple joint angles throughout the range of motion. This shift in approach would require more time and attention to detail in assessment and interpretation with this measurement tool, but may provide a more accurate assessment. With the added measurements, it is also possible that changes in specific curve characteristics could provide additional information that would have applied/clinical relevance. However, to be of use in the applied and clinical settings the parameters determined from the force-angle relationship should be reliable and have a low standard error of measurement (SEM). Therefore, the purpose of this study was to quantify the reliability and SEM of force-joint angle measurement methods in humans prior to damaging eccentric exercise,
evaluate changes in curve characteristics following eccentric exercise and examine the relationship of these changes to traditional markers of muscle damage. In addition, we want to determine whether or not force loss measured at a fixed joint angle is overestimated compared to measurements made at the optimal angle. We hypothesize that muscle force loss following eccentric exercise of the elbow flexors is greater in magnitude using a fixed angle measurement approach versus an optimal angle approach as a result of a shift in the optimal joint angle in subjects unaccustomed to eccentric exercise.

METHODS

Experimental Approach to the Problem

For this study, we used a within-subjects study design with 2 pre-eccentric exercise time points. The two baseline time points allowed us to examine the test-retest reliability of the curve fitting techniques and other muscle damage parameters, and served as a control for the post-eccentric exercise time points. After the baseline values were established, subjects performed the eccentric exercise task and then all dependent measures were reassessed immediately post, 1 day, 2 days, 4 days, and 7 days post-eccentric exercise to characterize changes over time (Figure 1-1). The results reported in this manuscript have not been reported prior, but are an extension of a larger study in which we examined changes in circulating matrix metalloproteinase-9 in response to eccentric exercise (McHugh & Tetro, 2003; Philippou et al., 2004; Chen et al., 2007; Brown & Donnelly, 2011; Madden et al., 2011). For the convenience of the reader and ease in understanding our results, we have either referenced our previous work or included select values within this manuscript for a few of the traditional markers of muscle damage that are relevant to both studies.
Subjects.

All subjects gave written, informed consent to participate in the study, which was approved by the University of Colorado - Boulder Institutional Review Board. Subjects were 18-29 year-old (21.5 ±3.7 S.D.), physically inactive males (n=14). Subjects were thoroughly screened for physical activity habits and health/disease status as described previously (Madden et al., 2011). Briefly, a physician at the CU Clinical Translational Research Center (CTRC) performed a medical history and physical exam to screen subjects for the determination of health/disease status, the presence of pathological conditions that could influence systemic levels of markers of regeneration and repair (muscle and liver enzymes), and the ability to safely perform the upper-extremity exercise task. Subjects were excluded from the study if they met or exceeded the ACSM’s definition of “physically active,” participating in moderate intensity aerobic physical activity for a minimum of 30 minutes per day, 5 days per week or vigorous activity for 20 minutes per day, 3 days per week (Pollock et al., 1998). Subjects were also excluded if they performed any weight lifting, resistance training, or similar activities in the previous 6 months. Subjects who passed the physical activity screening criteria and were included in the study, were told to avoid any exercise or physical activities, the use of ice and/or

Figure 1-1. Study timeline showing the general statistical analyses that were performed in the 7-visit study. The time point labels that are used in subsequent graphs are also provided.
anti-inflammatory medications, therapeutic stretching of the upper-extremities, and to maintain normal dietary habits for the duration of the study.

**Procedures**

Upon successful screening, subjects returned to the laboratory on 6 separate occasions to complete the experimental procedures at the following sampling times: Pre-injury 1 (Pre1), Pre-injury 2 (Pre2), Immediately- (IP), 1 day, 2 days, 4 days, and 7 days post-injury. The Pre1 and Pre2 time points were scheduled within 3-days of one another, and the Pre2 and IP sampling times occurred on the same day, when the eccentric exercise task was performed. During the first experimental visit (Pre1), baseline values were obtained for all of the dependent measures prior to any exposure to eccentric exercise-induced injury. In addition, a 1-repetition maximum (1-RM) test of the non-dominant elbow flexors was performed. The second visit was scheduled within 3 days of the first experimental visit and during the first half of this visit (Pre2) the dependent measures were reassessed for the purpose of obtaining test-retest reliability information about all of the measured parameters prior to the eccentric task. For the eccentric exercise task, subjects used their non-dominant arm to perform the eccentric portion only of an arm curl task on a custom built eccentric apparatus that has previously been described (Madden et al., 2011). They did this by holding onto a rigid handle affixed to a cable and pulley system, and lowering a weight corresponding to 120% of the maximal concentric 1-repetition maximum (1-RM) performed at the first experimental visit. The 120% maximal concentric 1-RM load is a greater load than what is typically used in protocols involving eccentric contractions of the elbow flexors (Edwards et al., 1977; Clarkson et al., 1986; Paddon-Jones & Quigley, 1997; Warren et al., 1999) and was chosen to ensure that the stimulus was robust. The eccentric task involved 6 sets of 10 repetitions each lasting 10 seconds with 2 minutes of rest between sets. Between each
repetition, the handle and cable were returned to the fully flexed elbow joint position by the investigator so that the subject was not performing the concentric portion of the task. All 60 repetitions were completed over the entire range of motion, even if a subject was unable to lower the weight at the controlled velocity. All dependent measures were reassessed at the following time points: Immediately post (IP), 1 day, 2 days, 4 days, and 7 days post-injury.

Venous blood was collected at each sampling time for the purpose of quantifying systemic markers of muscle injury over the study time course, and complete results are described in a separate publication (Warren et al., 1999; Madden et al., 2011) while only correlations to these parameters are presented here. Rating of perceived soreness was assessed with a computer-based Visual Analog Scale (VAS) controlled by the subject moving a 100-mm-long slider bar to indicate “least discomfort” versus “most discomfort.” This assessment was performed while the subject’s non-dominant arm was at rest and during 5-elbow flexion/extension motions. The subject was then seated on a stool at a custom-built (SuperStrut®, Thomas & Betts, Memphis, TN) strength test apparatus with the non-dominant arm supinated and supported at 90° of shoulder flexion in the sagittal plane by a padded platform. Maximal voluntary isometric strength was measured throughout a range (50-160°) of 11 joint angles measured in duplicate using a force transducer interfaced to an A-D converter (OMEGA Engineering, INC., Stamford, CT), and recording software (LabView, National Instruments, Austin, TX). Next, subjects performed a maximal concentric elbow flexion test on the same custom-built apparatus by holding onto a rigid handle affixed to a cable and pulley system to which weight could be incrementally added until the subject could no longer complete the concentric portion only of a single arm curl in the sagittal plane. Two minutes of rest were given between each consecutive attempt and the weight was lowered back to the starting position by the investigator. The highest weight achieved was
deemed the 1-RM and this 1-RM value was used in a calculation (Weight_{1-RM} \times 1.2) to determine the amount of weight to use in the next laboratory session for the eccentric exercise task, in accordance with load capacities used by other muscle damage researchers in the past (reviewed in (McHugh & Tetro, 2003; Philippou et al., 2004; Chen et al., 2007; Tiidus, 2008; Brown & Donnelly, 2011)). Range of motion (ROM) of the elbow joint was measured by positioning the fulcrum of a manual goniometer on the lateral epicondyle of the humerus and adjusting the goniometer arms to align with the lateral midline of the humerus and the lateral midline of the radius while the subject was seated at a stool. These anatomical references were marked with permanent marker for identification at follow-up visits. Limb circumference was measured at two different marked locations (mid-belly of the biceps brachii and at the elbow joint) using a soft tape measure.

After comparing the coefficient of determination ($r^2$) values for $2^{nd}$, $3^{rd}$, $4^{th}$, and $5^{th}$ order polynomial curves for all of the force-angle curves in the study, it was determined that $2^{nd}$ order polynomial curves produced the highest mean $r^2$ value and would be used for all subsequent analyses. Force-angle curves were constructed by fitting $2^{nd}$ order polynomial regression curves to the muscle force versus joint angle data points for individual subject visits. Optimal angle was determined by calculation of each curve’s vertex (General equation for $2^{nd}$ order polynomial: $ax^2+bx+c$; vertex: $-b/2a$). Curve fits were evaluated by the examination of the coefficient of determination ($r^2$) for the regression equation describing the curve. Criteria was established for excluding datasets for which a second order polynomial curve produced a non-physiological optimal angle value outside of the known range of motion for the joint ($<20^\circ$ or $>160^\circ$). Of the 18 who initially participated in this protocol, the datasets from 4 subjects met these exclusion criteria during at least one visit. These 4 subjects were excluded and the remaining 14 subjects
were included in the force-angle analysis. Alteration in curve height was quantified as the change in force between the vertex and an angle associated with the vertex + 30° along the descending limb of the force-angle curve. The aforementioned curve characteristics are labeled in Figure 1-2, which is experimental data obtained from one representative subject.

**Figure 1-2.** Muscle force – joint angle curve characteristics for one representative subject. Each curve represents one visit (solid curve = Pre2; dashed curve = IP). Curves were constructed for each subject by fitting 2nd order polynomial regression curves to 11 force-angle data points for each time point (General equation for 2nd order polynomial: \( ax^2 + bx + c \); vertex: \(-b/2a\)). Optimal joint angle was identified by calculation of the curve’s vertex (star symbol). A shift in the optimal joint angle was quantified by calculating the difference in the optimal angle along the x-axis between visits (thick dashed arrow). Curve height was defined as the change in force between the curve vertex and the vertex plus 30° along the x-axis (thin dashed arrow).
Statistical Analyses

The stability of the dependent variables was determined using paired t tests to compare means, the calculation of total error of measurement (TEM), and correlational analysis using the intraclass correlation coefficients (ICCs). Coefficient of variation (CV) and upper and lower 95% confidence limits (95% CI) were calculated using a statistical package that is available online (A New View of Statistics, Will G. Hopkins, 2009, <http://www.sportsci.org/resource/stats/index.html>). The PASW statistical package version 18.0 was used for all other statistical evaluations (SPSS Inc, Chicago, IL, USA). One-way (Fixed factor = laboratory session) repeated-measures analysis of variance (1-RM ANOVA) using Greenhouse-Geiser correction with Least Significant Difference (LSD) post-hoc analysis was performed on all continuous dependent measures. Data are presented as means ± standard error of measurement (SEM) unless otherwise stated.

RESULTS

Test-retest reliability from Pre1 to Pre2

A second order polynomial curve adequately described the force-angle relationship prior to the eccentric exercise intervention with no significant difference between the average $r^2$ values for Pre1 (0.879±0.021) and Pre2 (0.896±0.014) and an intraclass correlation (ICC) of 0.81. Typical error of measurement (TEM) and reliability correlations for study dependent measures for the pre-exercise time points (Pre1 vs. Pre2) are provided in Table 1-1, and ranged from 0.516 to 0.979, with the exception of creatine kinase activity. While the Pre1 to Pre2 CK activity correlation was only 0.115, CK levels were within the “normal” range of the assay on both days and day-to-day fluctuations were within the measurement error of the assay for both of these time points. Coefficient of variation (CV) values ranged from 4.4-12.7% for the dependent
measures describing the curve characteristics (optimal angle, force at optimal angle, and curve height) and raw TEM values are provided in Table 1-1.

### Table 1-1. Test-retest data table for pre-exercise time points (Pre1 vs. Pre2) including TEM, lower and upper 95% CI, and intraclass correlation coefficients.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TEM</th>
<th>LOWER 95% CI</th>
<th>UPPER 95% CI</th>
<th>CORRELATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAS</td>
<td>4.55 mm</td>
<td>3.30 mm</td>
<td>7.33 mm</td>
<td>0.516</td>
</tr>
<tr>
<td>ROM</td>
<td>3.16°</td>
<td>2.38°</td>
<td>4.75°</td>
<td>0.777</td>
</tr>
<tr>
<td>CK ACTIVITY</td>
<td>162.81 U/L</td>
<td>121.26 U/L</td>
<td>247.79 U/L</td>
<td>0.115</td>
</tr>
<tr>
<td>OPTIMAL ANGLE</td>
<td>4.47°</td>
<td>3.33°</td>
<td>6.81°</td>
<td>0.819</td>
</tr>
<tr>
<td>FORCE AT OPTIMAL ANGLE</td>
<td>5.21 N</td>
<td>3.78 N</td>
<td>8.39 N</td>
<td>0.977</td>
</tr>
<tr>
<td>CURVE HEIGHT</td>
<td>17.17 N</td>
<td>11.81 N</td>
<td>31.34 N</td>
<td>0.939</td>
</tr>
</tbody>
</table>

*Traditional Muscle Damage Parameters*

Figures for soreness, ROM, and CK responses are provided in a separate publication (Proske & Morgan, 2001; Chen et al., 2007; Madden et al., 2011). To summarize, soreness ratings were significantly increased (p<0.001) from both Pre1 and Pre2 values following the exercise task (IP, 1 day, 2 days, 4 days) and peaked at 1 day post-exercise (46.16 ±3.56 mm on a 0-100 mm scale). By 7 days post-exercise, soreness ratings were not significantly different from Pre1 or Pre2 values. There was a significant decrease (p<0.001) in ROM from Pre1 and Pre2 to all post-exercise time points, with the greatest changes occurring IP (-20.8 ±2.2°) and 1 day post (-18.8 ±2.3°). By 7 days post-exercise, ROM was still decreased by approximately 5°. CK
activity was significantly increased from Pre1 (136.5 ±48.3 U/L) to 4 days post-exercise (1939.4 ±943.2 U/L). By 7 days post-exercise, CK activity was not significantly different from Pre1 or Pre2 levels.

**Force loss and the force-angle relationship**

There was a significant (p=0.035) rightward shift in the optimal joint angle of force production from 88.0 ±11.88° to 98.4 ± 14.1° from Pre2 to IP with a restoration of optimal angle to Pre2 values by 1-day post (**Fig. 1-4a**) and for the remainder of the study. Interestingly, the r² value for the 2nd order polynomial curve fit was significantly decreased (p<0.001) from Pre2 (r²=0.896) to IP (r²=0.802) and 1-day post (r²=0.750) (**Fig. 1-4c**). By 2 days post, r² values were not significantly different from Pre2 values. Curve height was defined as the change in force between the vertex and an angle associated with the vertex + 30° along the descending limb of the force-angle curve (**Fig. 1-2**). There was a significant 39% decrease IP in the group mean curve height (**Fig. 1-4b**), resulting in a decrease in the height of the curve for many subjects (**Fig. 1-3**). Curve height remained decreased at 1 day and 2 days post-exercise, with a restoration to pre-exercise depth by 4 days post-exercise. The changes in curve height at IP, 1 day, and 2 days post-exercise suggest that the greatest force losses occur midway through the range of motion, at joint angles that result in the greatest force production. There was no significant correlation between shift in optimal angle and ROM, VAS, or CK activity. Nor was there a relationship between curve height and ROM, VAS, or CK activity.
Figure 1-3. Muscle force – joint angle relationship for all subjects at Pre2 (dashed curve) versus IP (solid curve). Force was normalized to each subject’s individual peak for ease of visualization of the general curve trends.

There was a significant (p<0.001) reduction in peak force measured at the optimal joint angle from Pre2 to multiple post-exercise time points. Force at the optimal angle was significantly reduced by 46.6% (SD ±11.3%) IP, 39.2% (SD ±17.4%) 1 day post, 31.0% (SD ±19.2%) 2 days post, and 17.2% (SD ±17.3%) 4 days post. By 7 days post-exercise, force at the optimal angle was restored to Pre2 levels. Force loss at a fixed angle of 90° of elbow flexion has been reported in a previous publication, but is also reported here for comparison with the optimal angle approach of quantifying force loss. In summary, there was also a significant reduction in peak force measured at a fixed angle of 90° of elbow flexion from Pre2 to multiple post-exercise time points. Force at 90° was significantly reduced by 47.9% (SD ±11.7%) IP, 39.4% (SD ±18.0%) 1 day post, 31.4% (SD ±19.6%) 2 days post, and 17.2% (SD ±17.4%) 4 days post. By 7
days post-exercise, force at 90° was restored to Pre2 levels. There was no significant difference at any time point in the group mean percent force loss using the optimal angle versus the fixed angle (90°) technique of quantifying loss of force.
Figure 1-4. Changes in force-angle curve characteristics following an upper extremity eccentric exercise task. Bars represent means ± SEM. * = significantly different from Pre-Ex, p<0.05. (A) There was a significant rightward shift (p=0.035) in the optimal joint angle of force production from 88.0 ± 11.88° to 98.4 ± 14.1° from Pre2 to IP with a restoration of optimal angle to Pre2 values by 1-day post. (B) There was also a significant decrease (p<0.001) in curve height from Pre2 to IP by 39%, resulting in a flattening appearance of the curve. (C) The $r^2$ value for the 2$^{nd}$ order polynomial curve fit was significantly decreased (p<0.001) from Pre2 ($r^2=0.896$) to IP ($r^2=0.802$) and 1-day post ($r^2=0.750$).
DISCUSSION

The results from this study indicate that the 2\textsuperscript{nd} order polynomial curve fitting technique is reliable prior to eccentric damage. The force-angle 2\textsuperscript{nd} order polynomial relationship is reproducible from day-to-day within a subject, as indicated by the low typical error of measurement (TEM) values and the high correlations (Pearson $r$) between the optimal angle, the force produced at the optimal angle, and the curve height measured at both pre-exercise time points (Table 1-1). The strength of the curve fit that describes the force-angle relationship decreased immediately- and 1-day post-eccentric exercise, as indicated by a significant decrease in the coefficient of determination ($r^2$ value) at these time points (IP: $r^2=0.802$; 1-day: $r^2=0.750$), and was restored by 2-days post-eccentric exercise ($r^2=0.848$). There were only 4 instances out of 98 curves analyzed where 2\textsuperscript{nd} order polynomial curves did not adequately describe the force-angle relationship following eccentric exercise, as indicated by a calculated vertex that was not within the physiological range of motion. These 4 curve data sets were excluded from further curve characteristic analyses, but may have actually provided additional support to the concept of a decrease in curve height following damage (since all 4 curves were from post-exercise time points) and a less distinct force-angle peak as a result of a more linear than curvilinear relationship.

Our work has also shown that in addition to a $\sim$17-47\% decrease in the magnitude of peak force production from immediately- to 4-days post eccentric exercise, several other force-angle curve characteristics also changed during this time period. We have repeated what others have shown in our finding of a rightward shift of about 10° in the muscle force-joint angle relationship immediately following damaging eccentric contractions (i.e., the curve vertex shifted and peak force was produced at longer muscle lengths). This finding is consistent with
several other human and animal studies that have associated the angle shift with the “popping” of sarcomeres (Proske & Morgan, 2001; Morgan & Proske, 2004; Butterfield & Herzog, 2005; Chen et al., 2007) and an increase in series compliance of the muscle. However, the change that we report is transient and the optimal joint angle for force production is restored to pre-eccentric exercise angle by 1-day post exercise. The timing of this curve characteristic shift and restoration suggests that muscle fatigue cannot be ruled out as a mechanism responsible as others have suggested (Butterfield & Herzog, 2005).

In our study, the flattening of the force-angle curves was very noticeable immediately post-eccentric exercise (Fig. 1-3), but also apparent at 1- and 2-days post-eccentric contractions. This curve change prompted us to develop a metric to describe curve height by quantifying the change in force between the vertex and an angle associated with the vertex plus 30 degrees along the descending limb of the curve. A change in curve height has not been reported by others, but at least one other investigator has alluded to a similar curve change in the form of a “counterclockwise rotation” of the force angle curve following eccentric contractions (Butterfield & Herzog, 2005).

It is difficult to interpret the meaning of the curve characteristic changes with eccentric exercise, but tempting to speculate that each change could have specific clinical relevance in the rehabilitation setting. For example, a shift in the optimal angle of force production could be an easily measureable sign of sarcomere damage, with the magnitude of the shift indicative of the magnitude of sarcomere damage. However, this relationship has not been confirmed with histological evidence in humans. In addition, our angle shift findings show a restored optimal angle by 1-day post-exercise, suggesting that a mechanism other than sarcomere damage alone might contribute to this shift (e.g., fatigue). There are several possible mechanistic explanations
for the more sustained changes in curve height (curve flattening), but this is the first time that curve height changes have been recognized and we did not specifically study the mechanism behind this change. Although we did not directly measure the mechanisms responsible for the curve characteristic changes in our study, we did examine curve characteristic correlations with traditional markers of muscle damage, such as soreness ratings (VAS), changes in range of motion, and creatine kinase activity. We did not find any correlations between either the shift in the optimal angle or the decrease in the curve height and any of the traditional markers of damage. The idea of associating a force-angle curve change with a physiological mechanism has the potential to provide a non-invasive metric to assess muscle in the rehabilitation setting, especially since each of the curve changes appears to recover, but additional work needs to be done to strengthen our understanding of the mechanisms associated with each change.

Our final goal in this study was to determine if the fixed angle technique of measuring force after damage results in an overestimation of force loss when compared to the optimal angle technique. If the muscle force produced across a range of joint angles is best described by a 2\textsuperscript{nd} order polynomial curve both before and after damaging exercise then it is mathematically likely that force loss estimations would be overestimated following an optimal angle shift as a result of the change in location of the curve vertex as it shifts to the right. We found that although there was a rightward shift in the curve vertex, there was no significant difference in the magnitude of force loss between the two techniques, which we have attributed to the curve flattening effect following eccentric contractions. Since there is no statistical difference between force loss estimates with either the fixed angle or optimal angle technique following damaging contractions, this finding suggests that either measurement technique can provide an adequate assessment of force loss for a clinician interested in quantifying this information.
PRACTICAL APPLICATIONS

There may be clinical value in associating each curve characteristic change with a physiological mechanism, but that has yet to be determined. Our work has provided a recommendation for how curve characteristics can be quantified and described, and the 2nd order polynomial approach is reliable. A link between changes in curve characteristics and specific muscle adaptations to damaging or other types of contractions could provide a useful, non-invasive metric for clinicians to determine optimal therapeutic approaches and appropriate timelines for the return to sport, but additional work needs to be done in this area. Unless a clinician is trying to gain a detailed and comprehensive understanding of changes in the muscle force-joint angle curve characteristics following damage, force loss measured at a fixed angle within 10 degrees of the pre-injury optimal angle and within the first 7 days following the insult seems to be a key parameter to continue to use. Measuring force loss at a fixed angle (as opposed to several angles within the ROM) may also be time saving for the clinician in an outpatient rehabilitation setting.
CHAPTER II

PLASMA MATRIX METALLOPROTEINASE-9 RESPONSE TO ECCENTRIC EXERCISE OF THE ELBOW FLEXORS

**ABSTRACT**

Recent efforts to establish a role for plasma matrix metalloproteinase-9 (MMP-9) as a marker of exercise-induced muscle damage have been inconsistent. Methodological and experimental design issues have contributed to confusion in this area. The purpose of this study was to use a damaging eccentric arm task to evaluate the relationship between activity-induced muscle damage and plasma MMP-9 levels in humans while controlling for physical activity history and quantifying day-to-day variability of the dependent variables. Fourteen physically inactive males performed 6 sets of 10 eccentric contractions of the elbow flexors at 120% of their voluntary concentric maximum. Soreness ratings, maximum voluntary isometric strength, range of motion (ROM), limb circumference, and plasma creatine kinase (CK) and MMP-9 levels were measured at 2 time points before, immediately after, and 1, 2, 4, and 7 days post-exercise. Changes in traditional markers of muscle damage mirrored patterns previously reported in the literature, but plasma MMP-9 concentration and activity measured by ELISA and gelatin zymography were unchanged at all time points examined. Plasma levels of the MMP-9 inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1) were also unchanged post-exercise. Finally, although mean MMP-9 levels were not significantly different between the two pre-exercise time points, the high total error of measurement and low day-to-day correlation suggest substantial within and between subject variability. Plasma MMP-9 levels are not a robust or reliable marker for eccentric exercise-induced damage of the elbow flexor musculature, though this may not preclude a role for MMPs in skeletal muscle remodeling in response to injury.
INTRODUCTION

Eccentric exercise, especially when the task is novel, produces muscle soreness and
strength loss as a secondary consequence of strain-induced muscle damage (Armstrong et al.,
1983a; Friden et al., 1983; Newham et al., 1983). Eccentric exercise-induced muscle damage has
been associated with disruptions to the muscle fiber contractile machinery (Fridén et al., 1981;
Friden et al., 1983; Yu et al., 2004), post-exercise infiltration of immune cells to the site of
injury (Armstrong et al., 1983b) and damage to, and degradation of, the muscle extracellular
matrix (ECM; (Brown et al., 1997; Kovanen, 2002; Kjaer et al., 2006). Increased degradation of
the ECM may contribute to decrements in force transmission (Gao et al., 2008) and may also be
a contributing factor to the recruitment of immune cells during the immediate post-exercise
inflammatory response (Adair-Kirk & Senior, 2008).

Matrix metalloproteinases, or MMPs, are secreted proteinases that degrade ECM proteins
and thus play a prominent role in the remodeling of the ECM during adaptive states (Sternlicht &
Werb, 2001). Expression and/or activity of several MMPs, and of the inducible gelatinase MMP-
9 in particular, is increased in rodent models of eccentric muscle damage (Koskinen et al., 2002).
Studies of systemic MMP-9 levels following eccentric exercise in humans have been more
equivocal (Koskinen et al., 2001a; Mackey et al., 2004) and the relationship between muscle
damage and systemic MMP-9 levels is still not clear. In human studies using lower limb
eccentric activity, MMP-9 was unchanged by downhill running at room temperature (Koskinen
et al., 2001a), but increased approximately equally (50%) by either downhill running in a cold
room (Koskinen et al., 2001a) or by high force isokinetic eccentric contractions (Mackey et al.,
2004) despite substantial differences in a different marker of muscle injury, plasma creatine
kinase levels, between these two tasks (Koskinen et al., 2001a; Mackey et al., 2004). Moreover,
the timing differed considerably in these studies, with MMP-9 increasing immediately after
downhill running in the cold (Koskinen et al., 2001a) and eight days following a single bout of
100 isokinetic eccentric contractions of the knee (Mackey et al., 2004). In these studies it is
unclear whether or not the protective repeated bout effect was controlled for because the subjects
in the downhill running study were described as physically active, young adult males (Koskinen
et al., 2001a), while the subjects in the knee extensor study were described as healthy, young
adult males and females (Mackey et al., 2004), without reference to specific training history or
physical activity screening criteria in either case. Finally, studies on the biological and
methodological variation in systemic MMP-9 levels, particularly in a well-defined physically
inactive but healthy young male population, are limited, and thus it is not clear whether MMP-9
may be a reliable index of post-exercise damage in humans. Systemic creatine kinase (CK) levels
have been traditionally used as blood indicators of muscle injury following damage, but the CK
response is highly variable in the general population (Ebbeling & Clarkson, 1989) and may not
correspond to the magnitude of muscle injury that has occurred (Warren et al., 1999; Clarkson &
Hubal, 2002).

The purpose of this study was to determine the effects of a well-characterized, highly
damaging eccentric arm task on systemic levels and activity of MMP-9 and on levels of its
inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1) in humans while rigorously controlling
for the confounding factors such as prior activity level associated with this experimental model.
We chose to utilize the eccentric arm task because of its frequent use in muscle damage research
as well as for its well-documented effects producing robust changes in traditional indices of
muscle damage such as maximal concentric force loss and, importantly, increased plasma
creatine kinase levels. In addition, the upper extremity musculature in physically inactive
individuals may be less protected from muscle damage by the repeated bout effect than the lower extremity musculature involved in locomotion. Direct comparisons between eccentric arm and lower limb tasks have amply demonstrated that arm tasks produce greater changes in force production and plasma creatine kinase (Jamurtas et al., 2005) as well as greater changes in intracellular signaling protein concentration (Thompson et al., 2003). We therefore hypothesized that MMP-9 activity would increase following a bout of upper-extremity eccentric exercise in physically inactive males.

METHODS

Subjects.

All subjects gave written, informed consent to participate in the study, which was approved by the University of Colorado (CU) Institutional Review Board. Subjects were 18-30 year-old, physically inactive males (n=14). Prior to participation in the exercise experiment, a physician at the CU-Boulder Clinical Translational Research Center (CTRC) performed a medical history and physical exam to screen subjects for the determination of health/disease status, the presence of pathological conditions that could influence systemic levels of markers of regeneration and repair (muscle and liver enzymes), and the ability to safely perform the upper-extremity exercise task. The physical exam included complete blood chemistries, and resting and exercise ECG during a maximal graded treadmill exercise test. In addition, subjects were asked about the nature, frequency, duration, and intensity of physical and occupational activities performed in the preceding 12 months through the use of an interviewer-administered Modifiable Activity Questionnaire (MAQ) (Kriska et al., 1990). Subjects were excluded from the study if they met or exceeded the ACSM’s definition of “physically active,” participating in moderate intensity aerobic physical activity for a minimum of 30 minutes per day, 5 days per week or
vigorous activity for 20 minutes per day, 3 days per week (Pollock et al., 1998). In addition, subjects were excluded if they performed any weight lifting, resistance training, or similar activities in the previous 6 months. Subjects who passed the physical activity screening criteria and were included in the study, were told to avoid any exercise or physical activities, the use of ice and/or anti-inflammatory medications, therapeutic stretching of the upper-extremities, and to maintain normal dietary habits for the duration of the study.

Experimental Protocol.

Following successful screening, subjects reported to the laboratory six times to complete the experimental procedures. These visits were scheduled at the same time of day and required subjects to adhere to the physical activity, anti-inflammatory, and dietary restrictions for the duration of the study. A summary of the study timeline is provided in Figure 2-1.
Figure 2-1. Schematic outlining the study visits. The Screening Visit included the informed consent, a medical history and physical including blood chemistry screening and a graded exercise test, and a physical activity inventory. The Baseline Visit was scheduled 7-10 days after the screening visit and served to determine baseline values for the dependent variables including plasma CK, MMP-9 and TIMP-1, soreness rating, range of motion (ROM), limb circumference, maximum voluntary isometric contraction force (MVIC). In addition, maximum voluntary concentric contractions (MVCC) were performed to determine a subject's maximal concentric force production. The Exercise Visit was scheduled 1-3 days after the Baseline Visit and involved repeating baseline dependent variable measures followed by the performance of the eccentric exercise task and subsequent measurement of the dependent variables. All remaining visits (24 hrs post, 48 hrs post, 4 days post, and 7 days post) involved measurement of the dependent variables.

The first visit served to determine baseline values for the dependent variables and determine each subject's maximal concentric strength. The second visit involved repeating baseline dependent variable measures followed by the performance of the eccentric exercise task and subsequent measurement of the dependent variables. Visits 4 to 7 involved measurement of the dependent variables. The dependent variables assessed were rating of perceived soreness, elbow flexor strength during a maximum voluntary isometric contraction of the non-dominant upper arm, range of motion (ROM), limb circumference, plasma CK activity, plasma MMP-9 activity, plasma MMP-9 concentration, and plasma TIMP-1 concentration.
For the baseline session, a blood sample was collected using standard phlebotomy procedures from the antecubital region of the dominant arm. A rigid wrist orthotic (Orthomerica Products, Inc., Newport Beach, CA) was then placed on the subject’s non-dominant arm for the remainder of each experimental session to prevent excessive wrist movement during the strength measurement and/or eccentric exercise injury task. Rating of perceived soreness was assessed with a computer-based Visual Analog Scale (VAS) controlled by the subject moving a 100-mm-long slider bar to indicate “least discomfort” versus “most discomfort.” This assessment was performed while the subject’s non-dominant arm was at rest and during 5-elbow flexion/extension motions. The subject was then seated on a stool at a custom-built (SuperStrut®, Thomas & Betts, Memphis, TN) strength test apparatus with the non-dominant arm supinated and supported at 90° of shoulder flexion in the sagittal plane by a padded platform. Maximal voluntary isometric strength at an angle of 90° of elbow flexion was measured using a force transducer interfaced to an A-D converter (OMEGA Engineering, INC., Stamford, CT), and recording software (LabView, National Instruments, Austin, TX). Next, subjects performed a maximal concentric elbow flexion test on the same custom-built apparatus by holding onto a rigid handle affixed to a cable and pulley system to which weight could be incrementally added until the subject could no longer complete the concentric portion only of a single arm curl in the sagittal plane. Two minutes of rest were given between each consecutive attempt to perform the concentric arm curl and the weight was lowered back to the starting position by the investigator. The weight associated with this task was deemed the 1-repetition maximum (1-RM). This 1-RM value was used in a calculation to determine the amount of weight to use in the next laboratory session for the eccentric exercise task. ROM of the elbow joint was measured by positioning the fulcrum of a manual goniometer on the lateral epicondyle of the humerus and adjusting the
goniometer arms to align with the lateral midline of the humerus and the lateral midline of the radius while the subject was seated at a stool. These anatomical references were marked with permanent marker for identification at follow-up visits. Limb circumference was measured at two different marked locations (mid-belly of the biceps brachii and at the elbow joint) using a soft tape measure.

For the eccentric exercise session, subjects returned to the lab at the same time of day and within 3 days of the Baseline session. Subjects were also reminded to adhere to the study procedures regarding exercise, diet, and anti-inflammatory medications. Prior to the exercise, all dependent parameters were re-assessed (Pre-Ex) for the determination of day-to-day (Baseline vs. Pre-Ex) biological variation. For the eccentric exercise task, subjects used their non-dominant arm to perform the eccentric portion only of an arm curl task. They did this by lowering a weight corresponding to 120% of the maximal concentric 1-RM performed at the Baseline session. The eccentric task involved 6 sets of 10 repetitions each lasting 10 seconds with 2 minutes of rest between sets. Between each repetition, the handle and cable were returned to the fully flexed elbow joint position by the investigator so that the subject was not performing the concentric portion of the task. All 60 repetitions were completed over the entire range of motion, even if a subject was unable to lower the weight at a controlled velocity. Immediately-post (Post-Ex) eccentric exercise, blood was collected again and soreness and strength were re-assessed. Subjects then returned to the lab on 4 additional occasions for follow-up visits at 1 day-, 2 days-, 4 days-, and 7 days-post eccentric exercise task. Each follow-up visit occurred at approximately the same time of day as the completion of the eccentric exercise session and included the collection of a blood sample and the assessment soreness and strength.
Blood sampling and storage.

Blood samples were drawn from an antecubital vein in the dominant arm by venipuncture at each visit and collected in lithium heparin-coated vacutainer tubes (BD®, Franklin Lakes, NJ) then immediately centrifuged for 15 minutes at 1500 x g. Blood plasma was separated and immediately frozen and stored at -20° C in small volume aliquots until used for the assays described, while avoiding more than 3 freeze-thaw cycles for any given aliquot as recommended by a recent publication (Souza-Tarla et al., 2005). All blood samples, basal and post-exercise, were collected, handled, and stored under identical conditions.

Gelatin zymography for MMP-9 activity.

Gelatin zymography was used to quantify MMP-9 activity in blood plasma as previously described (Allen et al., 2003). SDS polyacrylamide gels (10%) containing 1 mg/ml gelatin were casted and overlaid with a 4% stacking gel. Blood plasma was mixed with 1:1 volume sample buffer consisting of 50 mM Tris, pH 6.8, 2% SDS, 20% glycerol, and 0.2% bromophenol blue without reducing agent or heat. Electrophoresis was carried out at 100 volts until the dye front had reached the bottom of the gel. Gels were removed from the glass plates and washed in 2.5% Triton-X 100 three times for 15 minutes each time to remove SDS from the gel. The gels were then incubated at 37° C for 18 hrs in 50 mM Tris, pH 7.5, 10 mM CaCl₂. Gels were then stained with Coomassie brilliant blue for 30 min and de-stained with 40% methanol/10% acetic acid for 1 hr. Gels were imaged using a ChemiDoc-It imaging system (UVP, LLC., Upland, CA). Enzymatic activity was quantified for integrated density using ImageJ (NIH) software. Integrated density values for MMP-9 activity were normalized for each individual using his Baseline value.
*Plasma MMP-9 and TIMP-1 ELISA Assay.*

Total plasma MMP-9 and TIMP-1 were measured in duplicate by sandwich ELISA using commercially available kits (Quantikine, R&D Systems, Minneapolis, MN). Samples were prepared per the kit recommendations with a 40-fold plasma dilution for MMP-9 and a 100-fold plasma dilution for TIMP-1 into Calibrator Diluent RD5-10 and RD5P, respectively (Quantikine, R&D Systems, Minneapolis, MN), and measured using a microplate reader set to 450 nm with a correction of 540 nm.

*Creatine Kinase Activity Assay.*

A commercially available kit (Sigma-Aldrich, St. Louis, MO) was used for spectrophotometric analysis of creatine kinase activity with a peristaltic water pump maintaining a temperature of 37°C during analysis. Samples were prepared according to the kit and spectrophotometrically read at 340 nm for three minutes. Activity per minute was recorded and translated into U/L using a simple equation provided with the kit (Sigma-Aldrich, St. Louis, MO). Each sample was quantified in duplicate. Control samples (DC-TROL level 1 & 2, Sigma Aldrich, St. Louis, MO) were also analyzed each day creatine analyses were performed.

*Statistics.*

The stability of the dependent variables was determined using paired t-tests to compare means, the calculation of total measurement error (TEM), and correlational analysis using the Pearson r value. Coefficient of variation (CV) and upper and lower 95% confidence limits (95% CI) were calculated using a statistical package that is available online (A New View of Statistics, Will G. Hopkins, 2009, <http://www.sportsci.org/resource/stats/index.html>). The PASW statistical packages version 17.0 was used for all other statistical evaluations (SPSS Inc, Chicago,
IL, USA). One-way (Fixed factor = laboratory session) repeated-measures analysis of variance (1-RM ANOVA) using Greenhouse-Geiser correction with Least Significant Difference (LSD) post-hoc analysis was performed on all continuous dependent measures. Data are presented as means ± SEM unless otherwise stated.

RESULTS

Indices of Muscle Damage

In total, 18 subjects were recruited for this study; however, dependent measures from all time points were assessed in just 14 of 18 subjects, and only these 14 are reported here. Two subjects did not show up for their last visit and blood samples were difficult to collect from 2 other subjects, so measures from these subjects were excluded from the RM-ANOVAs. Individuals were 18 to 29 years in age (21.4 ± 3.2 years), 180.43 ± 5.7 cm in height, and 70.81 ± 10.3 kg in mass. A schematic of the study timeline and parameters measured at each visit is provided in Figure 2-1.

Soreness, strength, and CK responses to the eccentric arm task are summarized in Figure 2-2. There was no significant difference in soreness from Baseline (1.80 ± 3.10 mm) to Pre-Ex (4.71 ± 8.82 mm). Although the p-value was close to being significant (p=0.092), the mean soreness values were all on the very low end of a 100-mm scale at both of the pre-exercise time points, and the correlation between the two values was very strong (r=0.885). Soreness ratings were significantly increased from both Baseline and Pre-Ex values following the exercise task (Post-Ex, 1 day, 2 day, 4 day) and peaked at 1 day post-exercise (Figure 2-2A). By 7 days post-exercise, soreness ratings were not significantly different from Baseline or Pre-Ex values.
There was no significant difference in maximum isometric strength from Baseline (126.53 ± 42.87 N) to Pre-Ex (119.70 ± 36.45 N; p=0.110), and the values were strongly correlated (r=0.919). The TEM for the pre-exercise values was 12.13 N (95% CI: 9.11-18.19 N), and the coefficient of variation (CV) was 10.9% (95% CI: 8.1-16.7%). Maximum isometric elbow flexion force was significantly decreased from Pre-Ex values at all time points following the exercise task, with the peak of maximal force loss of approximately 51% and 44% occurring at the Post-Ex and 1 day-post time points, respectively (Figure 2-2B). By 7-days post-exercise, maximum isometric force remained significantly decreased compared to Baseline and Pre-Ex strength, although there was a trend toward a restoration to Baseline values.

There was no significant difference in CK activity from Baseline to Pre-Ex (p=0.615), but the Baseline and Pre-Ex values were poorly correlated (r=0.115), TEM for CK activity was 162.81 U/L (95% CI: 121.26-247.79 U/L), and the coefficient of variation (CV) was 112.3% (95% CI: 75.2-214.4%). CK activity was highly variable (Figure 2-3), but significantly increased 15-fold from Baseline to 4 days post-exercise. By 7-days post-exercise, CK activity was not significantly different from Pre-Ex or Baseline levels (Figure 2-2C).
Figure 2-2. Traditional indicators of muscle damage across multiple time points following an upper extremity eccentric exercise task. (A) Visual analog scale soreness rating during 5 repetitive elbow flexion/extension tasks was significantly increased from both Baseline and Pre-Ex values at the Post-Ex, 24 hr, 48 hr, and 4 day time points. By 7 days post-exercise, soreness rating was not significantly different from Baseline or Pre-Ex values. (B) Elbow flexor maximal voluntary isometric force measured at an arm angle of 90° of elbow flexion was significantly decreased from both Baseline and Pre-Ex values at all time points following the eccentric exercise task, but there was a trend toward a restoration of force by 7 days post-exercise. (C) Plasma creatine kinase (CK) activity (u/l) was significantly increased from Baseline and Pre-Ex values at 4 days post-exercise. Bars represent means ± SEM. * = significantly different from Baseline and Pre-Ex, p<0.05.
Figure 2-3. Variation in post-exercise plasma Creatine Kinase (CK) levels. Lines represent the individual changes in plasma CK activity. The group mean values are represented by the shaded bars. CK activity was highly variable, but significantly increased 15-fold from Baseline to 4 days post-exercise.

ROM and limb circumference responses to the eccentric arm task are summarized in Figure 2-4. There was no significant difference in ROM from Baseline to Pre-Ex (p=0.171) and the two pre-exercise time points were strongly correlated (r=0.777). The TEM for the pre-exercise ROM values was 3.16° (95% CI: 2.38-4.75°) and the CV was 2.6% (95% CI: 2.0-4.0%). There was a significant decrease (p<0.05) in ROM from pre-exercise to all post-exercise time points and by 7 days post-exercise, ROM was still decreased by approximately 5° (Figure 2-4A).

There was no significant difference in limb circumference at either the belly of the biceps brachii (p=0.898) or the elbow joint (p=0.302) from Baseline to Pre-Ex and the pre-exercise time points were strongly correlated for both parameters (r=0.990 and r=0.988, respectively). The
TEM for the biceps belly pre-exercise values was 3.29 mm (95% CI: 2.47-4.94 mm) and the CV was 1.1% (95% CI: 0.8-1.7%). The TEM for the elbow joint pre-exercise values was 2.26 mm (95% CI: 2.00-3.99 mm) and the CV was 1.1% (95% CI: 0.8-1.6%). Arm circumference at the mid-belly of the biceps brachii was significantly increased (p=0.020) from pre-exercise values by +6.58 mm at 48 hrs post exercise and by +6.47 mm at 4 days post exercise. By 7 days post exercise, mid-belly arm circumference was not different from pre-exercise values. Arm circumference at the elbow joint was significantly increased (p<0.05) at all post-exercise time points and was maximal at 7 days post exercise (+10.16 mm) (Figure 2-4B).

Figure 2-4. Changes in range of motion (ROM) and limb circumference across multiple time points following an upper extremity eccentric exercise task. (A) Range of motion decreased significantly at all post-exercise time points. Bars represent means ± SEM. * = Significantly different from Baseline and Pre-Ex. (B) Limb circumference measured at the mid-belly of the biceps brachii (light bars) increased significantly from both Baseline and Pre-Ex values at 48 hrs and 4 days post exercise. Limb circumference measured at the elbow joint (dark bars) increased significantly from both Baseline and Pre-Ex values at all post-exercise time points with a peak increase at 7 days post exercise. Bars represent means ± SEM. * = Mid-belly measurements significantly different from Baseline and Pre-Ex, p<0.05. # = Elbow joint measurements significantly different from Baseline and Pre-Ex, p<0.05.
**Baseline and Post-Exercise MMP-9 Levels**

Basal plasma MMP-9 concentration ranged from 13.12 – 150.75 ng/ml with mean values of 59.10 ng/ml at Baseline and 59.27 ng/ml at Pre-Ex. A literature search for studies examining plasma MMP-9 values in control subjects from a variety of ages and ethnicities using the same kit and blood sampling methodology as the present study yielded over 25 publications with a range of MMP-9 values from 17.2 (Castellano *et al.*, 2008) to 156.7 ng/ml (Gai *et al.*, 2009) with most values clustered within 40-100 ng/ml. Thus the baseline values of ~60 ng/ml reported in the present study are well within both the range of values reported by the manufacturer (R & D Systems) for control populations (13-105 ng/ml) and the range of published values by other laboratories.

There was no significant difference in MMP-9 concentration from Baseline to Pre-Ex (p=0.721). The TEM for the pre-exercise values was 35.45 ng/ml (95% CI: 26.40 – 53.95 ng/ml) and the CV was 75% (95% CI: 51.0-134.3%). The correlation between the Baseline and Pre-Ex MMP-9 levels was 0.100. **Figure 2-5A** shows the individual changes in plasma MMP-9 concentration for the subjects in this study and illustrates the wide inter-subject variation in basal MMP-9 levels from day-to-day. Since all time point samples for each subject were handled identically and analyzed on the same plate, these day-to-day differences likely reflect biological and not methodological variation. Two subjects showed increases in MMP-9 concentration between the Baseline and Pre-Ex time points, from 20-40 ng/ml to 100-140 ng/ml (**Figure 2-5A**). Conversely 3 subjects showed decreases in plasma MMP-9 concentration from ~100-150 ng/ml to 30-80 ng/ml (**Figure 2-6A**). The rest of the subjects showed only modest changes in plasma MMP-9 concentration from Baseline to Pre-Ex (**Figure 2-6A**).
Figure 2-5. Variation in pre-exercise plasma MMP-9 (A) and TIMP-1 (B) levels. Lines represent the individual changes in plasma MMP-9 (A) and TIMP-1 (B) concentration for two resting time points (Baseline and Pre-Ex) and all post-exercise time points (Post-Ex, 24 hr, 48 hr, 4 day, and 7 day). The group mean values are represented by the shaded bars. There was no main effect for sampling time for MMP-9 concentration ($p=0.375$) or for TIMP-1 concentration ($p=0.106$), but this figure illustrates the wide inter-subject variation in basal MMP-9 and TIMP-1 concentration from day-to-day (Baseline vs. Pre-Ex) and how that compares with the post-exercise changes.
There was no significant post-exercise change in circulating levels of MMP-9 as measured by gelatin zymography (p=0.294) (Figure 2-6A) or in either MMP-9 or TIMP-1 as measured by ELISA over the time course of the study (Figures 2-6B and 2-6C). There was a trend toward a decrease in plasma TIMP-1 activity from Pre-Ex (96.62 ± 23.03 ng/ml) to Post-Ex (80.06 ± 28.10 ng/ml), but the difference was not significant (p=0.106) and was heavily influenced by one individual (Figure 2-5B).
Figure 2-6. Plasma MMP-9 concentration and activity and TIMP-1 concentration post-exercise. Plasma MMP-9 activity (fold change relative to Baseline) quantified by gelatin zymography and (B) MMP-9 concentration (ng/ml) quantified by ELISA at multiple time points following eccentric exercise of the elbow flexors in humans. (C) TIMP-1 concentration quantified by ELISA at multiple time points following eccentric exercise of the elbow flexors in humans. There was no main effect for sampling time for MMP-9 activity (p=0.294) or concentration (p=0.375) or TIMP-1 concentration (p = 0.106). Bars represent means ± SEM.
DISCUSSION

The results from the present study demonstrate that plasma MMP-9 is not a reliable or robust systemic marker for exercise-induced muscle damage or repair following an eccentric arm task in humans. As mentioned above, we chose this eccentric arm task in part because despite the smaller volume of muscle involved (arm flexors vs. knee extensors), this type of task has been demonstrated to produce greater changes in damage indices, most notably in another systemic marker of muscle damage, plasma creatine kinase, than lower limb tasks of the same relative intensity (Thompson et al., 2003; Jamurtas et al., 2005). If systemic MMP-9 levels are indeed a robust and reliable measure of muscle damage, then we predicted that the eccentric arm task used here would produce an equal or even greater increase in plasma MMP-9 levels than those reported previously for lower limb eccentric tasks (Koskinen et al., 2001a; Mackey et al., 2004). However, while changes in traditional markers of muscle damage (soreness rating, CK, peak isometric force) were significant and indicated that the upper-arm eccentric exercise task was indeed a damaging stimulus, neither plasma MMP-9 concentration nor activity was significantly changed at any post-exercise time point.

Moreover, while mean pre-exercise plasma MMP-9 levels were within the expected range of published values (Castellano et al., 2008; Gai et al., 2009), individual variation in pre-exercise plasma MMP-9 levels was high both across- and within-subjects (Figure 2-4). Other investigators have also reported a large range of basal systemic MMP-9 values. Specifically, Tayebjee and colleagues reported that resting blood plasma MMP-9 levels to ranged from 17 to 115 ng/ml in their subjects (Tayebjee et al., 2005). Similarly, in a human MMP-9 study involving eccentric contractions of the knee extensors, mean pre-exercise serum MMP-9 was 112 ng/ml with a standard deviation of ± 42 ng/ml (Mackey et al., 2004). A key strength of the
present work is the fact that we examined two pre-exercise time points for all measures, including plasma MMP-9 levels, and thus were able to monitor day-to-day fluctuations in plasma MMP-9 levels within subjects independent of the exercise stimulus. Our results demonstrate that much of the variation in plasma MMP-9 levels appears to be due to wide day-to-day variation within subjects, with several subjects showing dramatic changes (increases or decreases) in plasma MMP-9 levels between the Baseline and Pre-Ex visits, which were always less than 3 days apart (Figure 2-4A).

Part of the large variation in day-to-day MMP-9 activity observed in any study can be attributed to biological variation and part to methodological variation. As mentioned above, in the present study all samples for a given subject were processed and frozen identically and analyzed at the same time on the same ELISA plate. Thus differences in plasma MMP-9 values between the Baseline and Pre-Ex time points for a given subject were unlikely to reflect major methodological differences in sample storage or analysis. However, because each sample in the present study was assayed in duplicate, an estimate of the intra-sample methodological variability can be made. The mean CV for plasma MMP-9 activity measured between duplicates in the present study was 7.79%. This CV value represents a reasonable estimate of the methodological variation associated with the assay performed, but it is only a small contributor to the CV between the Baseline and Pre-Ex (75%), and thus much of the variability within subjects is likely due to biological variability.

It is not clear what might have caused the high biological variability between the two pre-exercise time points. Polymorphisms exist in the MMP-9 promoter, but are not linked to plasma MMP-9 variation in healthy subjects (Demacq et al., 2008). The acute effect of diet on plasma MMP-9 levels has not been adequately explored, and thus it is not clear whether day-to-day
variations in diet may have explained the differences observed in plasma MMP-9 in the present study. In addition, subjects were asked to maintain their regular diet and came in at the same approximate time for each visit, and thus it is unlikely that changes in diet or alimentation account for the majority of the variability reported here. Similarly, subjects were also screened so that they had not performed upper-extremity weight-lifting, resistance training, or similar activities in at least the previous 6 months and were told to avoid any exercise or physical activities, the use of ice and/or anti-inflammatory medications, and/or therapeutic stretching of the upper-extremities for the duration of the experiment. It therefore seems unlikely that differences in day-to-day physical activity levels accounted for an appreciable amount of the biological variance in pre-exercise plasma MMP-9 levels reported here. Thus at the present time there is not a clear explanation for the high biological variability that contributes to the remainder of the TEM for pre-exercise plasma MMP-9 levels within subjects.

Creatine kinase has often been criticized as a marker for exercise-induced muscle injury because it has been shown to be highly variable in the general population (Ebbeling & Clarkson, 1989; T & T, 1989) and because it may not appropriately reflect the magnitude of muscle damage that has occurred (Warren et al., 1999; Clarkson & Hubal, 2002). Moreover, like the pre-exercise plasma MMP-9 levels reported here, basal blood creatine kinase levels can also vary substantially across subjects (Strømme et al., 2004). However, in the present study the experimental effect of damaging arm exercise caused a robust and significant CK response, as unlike plasma MMP-9 levels, peak plasma CK levels exceeded the methodological and biological variability of this parameter as demonstrated by the statistically significant 15-fold increase that we report here at 4 days post-exercise. Thus despite similar limitations in terms of pre- and post-exercise variance, eccentric arm exercise was sufficient to produce an increase in
systemic CK but not MMP-9 levels. And while differences in assay sensitivity may contribute to some of this discrepancy, it nevertheless supports the conclusion that plasma MMP-9 levels are not a robust indicator of muscle damage. Biopsy studies and/or microdialysis may provide better insights into whether local changes in MMP-9 expression occur within the muscle following damaging exercise, but these are considerably more invasive and time consuming than blood collection.

Despite the high variability in pre-exercise systemic MMP-9 levels, the lack of a post-exercise systemic MMP-9 response in the present study was unexpected because of previous work demonstrating a significant increase in MMP-9 levels following eccentric activity of the lower limb(s) (Koskinen et al., 2001a; Mackey et al., 2004). However, the significant systemic increases in MMP-9 that have been reported by others occur at inconsistent time points, are sometimes dependent upon the room temperature where the exercise is performed (Koskinen et al., 2001a), and are within the observed range of day-to-day variability that we report here. These inconsistencies are likely one of the reasons Koskinen and colleagues concluded that serum levels of MMP-9 do not sensitively respond to exercise induced muscle damage (Koskinen et al., 2001a), which is consistent with the findings of the present study. We tried to maximize our likelihood of observing a systemic MMP-9 response by incorporating rigorous controls into our subject selection and study design. For example, we attempted to control for the well-documented “repeated bout effect” (Byrnes et al., 1985), in which prior bouts of eccentric exercise reduce the magnitude of muscle soreness and damage in subsequent bouts, by recruiting subjects who had not participated in any physical or occupational activities with their upper-extremities for a minimum of 6 months prior to the experiment, which was not explicitly described in other similar studies (Koskinen et al., 2001a; Mackey et al., 2004). In addition, we
chose to produce injury in the upper- versus lower-extremity muscles because the arm muscles, particularly in the non-dominant arm, tend to get used less frequently than those of the lower limb which are used daily for locomotion, going down stairs, etc. By selecting an inactive subject population and an appropriate task, we felt that we would minimize the effects of the repeated bout effect in a way that would maximize our likelihood of observing a systemic MMP-9 response despite the high variance in basal MMP-9 levels, yet we still did not observe an increase in systemic MMP-9 following eccentric exercise.

It is not clear why eccentric arm exercise failed to produce an increase in systemic MMP-9 levels in the present study while at least one study using the lower limbs elicited increases in systemic MMP-9 levels following exercise (Mackey et al., 2004). One possibility is that the single arm eccentric task used here, while damaging, did not involve sufficient tissue volume to elicit systemic increases in MMP-9 levels. However, given that systemic CK levels are typically greater following an eccentric arm task compared to a leg task of the same relative intensity (Jamurtas et al., 2005), this seems unlikely. Another possibility is that the duration of our study was too short, since Mackey et al. reported a significant increase in MMP-9 levels at 8 days post-exercise. However, given the dramatic and significant changes occurring to the traditional markers of muscle damage (soreness, arm circumference, ROM, maximal force production, CK) within the 7 day time range used in this study, we feel that 7 days should have been more than ample time to detect a change in MMP-9. Indeed, most of these other damage markers had resolved back to baseline values by 7 days, and thus it seems unlikely that a change in MMP-9 would occur outside this time frame. In addition, we observed no trend towards an increase in MMP-9 levels at 7 days post-exercise, which might suggest that a significant change might have occurred at 8 days or some later time point. Another possibility is that changes in systemic
MMP-9 levels may be greater for low-force, high repetition activities like running. Consistent with this, in a human study, marathon running produced an increase in serum MMP-9 immediately following a race (Saenz et al., 2006). This possibility might explain why MMP-9 mRNA and activity was increased in humans after a single bout of cycling exercise (Rullman et al., 2007) and a single bout of knee extensions (Rullman et al., 2009), two tasks that are typically not associated with muscle damage. Third, it is possible that differences in ECM composition and/or turnover exist between the upper and lower limb musculature, but if this is the case it has not been well documented scientifically. Fourth, differences in leakage rates into the systemic vasculature and/or local clearance of MMP-9 between the upper and lower extremities may also exist though, again, scientific confirmation of these is lacking.

Another possibility is that the eccentric arm exercise task, while damaging, may not be sufficient to cause alterations to the muscle ECM. In a recent review by Butterfield, the distinction between exercise-induced muscle damage and severe strain-induced muscle injury was emphasized, and the author pointed out that in vivo exercise protocols often involve less mechanical strain to muscle fibers when compared with in vitro or in situ muscle damaging protocols typically done in rodents (Butterfield, 2010). It is thus possible that MMP activity is increased and detectable in the systemic circulation only following a protocol that more closely resembles traumatic muscle injury, rather than exercise-induced injury. In studies using severely damaging interventions such as cardiotoxin injection (Kherif et al., 1999), ischemia-reperfusion (Roach et al., 2002), crush (Zimowska et al., 2008) or denervation (Chattopadhyay et al., 2007), an increase in muscle MMP-9 activity is almost always reported. Similarly, pathological muscle diseases resulting in a chronic state of muscle damage and inflammation are also associated with increased basal levels of MMP-9, both locally (Kieseier et al., 2001; Schosser et al., 2002) and
systemically (Koskinen *et al*., 2001a). Marathon running is associated with increases in systemic MMP-9 levels (Saenz *et al*., 2006), but in this case the ground reaction force produced by the feet over an extended period of time may induce additional traumatic injury to muscles or other limb structures (bones, tendons, ligaments) that result in greater connective tissue damage. In the present study, the fact that the eccentric arm exercise task did not result in a detectable systemic MMP-9 response may indicate that *in vivo* eccentric arm exercise is not, or is only minimally disruptive to, the muscle ECM.

While the results from the present study suggest that systemic MMP-9 was not changed after an upper-extremity eccentric task, there may still be a role for MMP-9 in muscle damage and repair. As mentioned above, levels of MMP-9 activity may have been increased locally within the tissue and been involved in immune cell infiltration, connective tissue repair/remodeling, or both. Secondly, other MMPs, and in particular MMP-2, have also been implicated in the remodeling of the ECM proteins surrounding skeletal muscle (Kherif *et al*., 1999; Koskinen *et al*., 2001a; Sternlicht & Werb, 2001). Though MMP-2 is constitutively expressed (Sternlicht & Werb, 2001), some investigators have reported changes in local (Koskinen *et al*., 2001b) and systemic MMP-2 following eccentric exercise (Koskinen *et al*., 2002). By visual inspection of the gelatin zymograms from our eccentric arm exercise experiment, there was no indication that MMP-2 activity was changed at any specific time point throughout the experiment reported here (data not shown).

This study was designed to evaluate systemic MMP-9 levels as an index of muscle damage following eccentric contractions. Through a series of experiments, we have concluded that plasma MMP-9 is not a robust systemic index for eccentric arm exercise-induced injury in humans. Whether or not MMP-9 or other MMPs might be involved in the cellular adaptations
that occur at the level of the tissue in response to eccentric exercise is not clear at this time. In future studies, establishing a relationship between the magnitude of disruption to the muscle ECM and the tissue MMP-9 response may provide more support for the role of MMPs in skeletal muscle injury and repair.
CHAPTER III

PLASMA MATRIX METALLOPROTEINASE-9 RESPONSE TO DOWNHILL RUNNING IN HUMANS


ABSTRACT

Matrix metalloproteinase-9 is a proteolytic enzyme capable of degrading proteins of the muscle extracellular matrix. Systemic levels of MMP-9 or its inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1), have the potential to serve as blood markers of exercise-induced muscle damage. The purpose of this study was to determine if an eccentrically-dominated task, downhill running (DHR), produces changes in plasma MMP-9 or TIMP-1 and examine the relationship between MMP-9/TIMP-1 levels and indirect indicators of muscle damage. Subjects were sedentary (SED, n=12) or had a history of concentrically-biased training (CON, n=9). MMP-9 and TIMP-1 were measured before (Pre-Ex), immediately after (Post-Ex), and 1-, 2-, 4-, and 7-days post-DHR (-10°), and compared to discomfort ratings, creatine kinase activity, and strength loss. At 1-day Post-Ex, discomfort increased (5.6±7.8 to 45.5±19.9 mm; 0-100 mm scale), strength decreased (-6.9±1.6%), and CK increased (162.9±177.2%). MMP-9 was modestly but significantly increased at Post-Ex in both CONC and SED (32.7±33.6%) and at 4-days in SED (66.9±88.1%), but individual responses were variable. There were no correlations between MMPs and discomfort ratings, plasma CK or strength. Plasma MMP-9 changes may be detectable in the systemic circulation after DHR, but changes are small and do not correspond to other markers of damage.
INTRODUCTION

Skeletal muscle damage is characterized by physical disruption to sarcomeres (Yu et al., 2004), infiltration of immune cells (MacIntyre et al., 2000), and eventual cell death (Yu et al., 2004). These events are accompanied by a perception of muscle soreness (Clarkson & Hubal, 2002), loss in muscle strength (Warren et al., 1999), and an increase in intracellular muscle proteins and enzymes in the circulation (Schwane et al., 1983; Sorichter et al., 1999). In addition to disruption of the contractile elements within muscle fibers, there is evidence that muscle damage and repair are accompanied by alterations to the extracellular matrix (ECM) surrounding the muscle fibers as well. The ECM is a meshwork of proteins that may provide more than structural support to the muscle (Chen & Li, 2009). There is evidence to suggest that modifications to the ECM may be necessary for migration of immune cells to injured fibers (Reichel et al., 2008), liberation of growth factors, satellite cells, and/or chemokines (Sternlicht & Werb, 2001), and activation and migration of satellite cells to replace damaged fibers (Nishimura et al., 2008; Chen & Li, 2009).

Modifications to the ECM are accomplished by the degradation and/or reassembly of ECM proteins (Sternlicht & Werb, 2001; Chen & Li, 2009). The composition of the ECM surrounding skeletal muscle dictates the type of proteolytic, matrix-degrading enzymes that may be present and active in the tissue during times of increased remodeling (Sternlicht & Werb, 2001). A main component of the muscle ECM, and the basal lamina surrounding each muscle fiber in particular, is type IV collagen (Sternlicht & Werb, 2001), which is the primary substrate for matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9). There is evidence to suggest that in skeletal muscle, MMP-2 is constitutively expressed while MMP-9 expression is inducible (Guérin & Holland, 1995) and MMP-9 expression may be particularly increased during periods
of robust matrix remodeling (Sternlicht & Werb, 2001). Increases in MMP-9 levels have been reported in animal models of muscle damage, including muscular dystrophy and cardiotoxin injection (Kherif et al., 1999). There are several clinical conditions associated with muscle breakdown that have also been associated with increases in MMP-9, including inflammatory myopathies (Choi & Dalakas, 2000) and denervation injuries (Schoser et al., 2002). The animal and human findings led to the idea that plasma MMP-9 might serve as a valuable, non-invasive biomarker that changes in proportion to the amount of muscle damage that has occurred. A clinically valuable blood marker of muscle damage would be one that: 1) is mechanistically involved in the cellular sequence of events that characterize skeletal muscle damage and repair; 2) is elevated in the systemic circulation when muscle is damaged; and 3) is increased in proportion to the amount of damage that has occurred. Such a marker has yet to be identified, but the proteins associated with the muscle extracellular matrix and its turnover have become interesting prospects with MMP-9 serving as a lead candidate.

In the context of exercise-induced muscle damage, increases in MMP-9 have been reported following isolated limb eccentric exercise tasks in both animals and humans. These models include forced lengthening contractions in rats (Koskinen et al., 2002), and high-force eccentric quadriceps contractions in human subjects (Mackey et al., 2004). However, we recently reported that plasma MMP-9 is highly variable in sedentary human subjects from day-to-day and there is no significant systemic increase following an eccentric elbow flexor task that has been well characterized as damaging (Madden et al., 2011). Conversely, our lab has used a downhill treadmill model of eccentric exercise in mice and we have shown direct signs of muscle damage to the tissue and increases in MMP-9 in the circulation (Mehan, 2013). Our downhill treadmill findings in mice encouraged us to pursue an equivalent model in humans, with the thought that
downhill running may be a unique stimulus to muscle and ECM that is different from the
stimulus delivered by an isolated muscle task. Downhill running is a cyclical, whole-body
motion that also involves a larger muscle mass than the isolated elbow flexor model that we have
used previously, and the role of muscle mass on the systemic MMP-9 response to eccentric
exercise has not been well characterized.

Only one group has examined changes in circulating MMP-9 following downhill
treadmill running in humans (Koskinen et al., 2001a). This group reported no change in serum
MMP-9 in physically-active subjects that ran in a 22°C ambient temperature room, but an
increase in the immediately post-exercise time point in the subjects who ran in a room set to a
temperature of 5°C. While a potential explanation for the temperature effect was provided, there
were several methodological limitations that could have also contributed to the disparate
response between the groups. Specifically, the subjects that were included had a prior history of
exercise training, which could potentially provide protection from muscle damage through the
repeated bout effect (Byrnes et al., 1985). In addition, blood serum was used to assess MMP-9
levels, and there is evidence to suggest that MMP-9 levels can be artificially elevated when the
serum is examined instead of the blood plasma (Jung et al., 2001a). We modeled our current
study after this only other human downhill running MMP-9 study, but with more emphasis on
selection of a subject population that is susceptible to muscle damage and on using
recommended pre-analytical techniques for blood sampling, and with less emphasis on the role
of ambient temperature on the MMP-9 response. We included two subject groups in the current
study: subjects with a history of concentrically-dominated exercise training (i.e., cycling,
swimming), and subjects who had no history of exercise training (i.e., “sedentary”). While there
is evidence to suggest that both of these subject groups may be susceptible to lengthening-
induced muscle damage (Ploutz-Snyder et al., 1998; Whitehead et al., 1998; Gleeson et al., 2003), the groups differ in their exercise-training history. This is the first study examining plasma MMP-9 levels in two populations of subjects with an increased susceptibility to muscle damage following a downhill running task. The purpose of this study was to examine changes in systemic MMP-9 and TIMP-1 levels following downhill treadmill running in two subject populations with a potentially increased susceptibility to lengthening-induced muscle damage. Based on the results from our mouse downhill running experiments and the improved subject selection criteria employed in this study, we hypothesized that downhill running would result in an increase in circulating markers of ECM remodeling (MMP-9, TIMP-1). Since CK activity and discomfort ratings have been criticized for not necessarily representing the magnitude of damage that occurs to the tissue [reviewed in (Warren et al., 1999)], we are not confident that a correlation will exist between these variables, but there is more support for a potential correlation with strength loss.

METHODS

Subjects

All subjects gave written, informed consent to participate in the study, which was approved by the University of Colorado (CU) Institutional Review Board and conducted in accordance to the principles of the Declaration of Helsinki and the ethical standards of the International Journal of Sports Medicine (Harriss & Atkinson, 2011). Subject eligibility criteria for enrollment into the study was strictly enforced, especially with regards to physical activity history. To confirm physical activity history, the researcher interviewed each subject about the nature, frequency, duration, and intensity of all physical and occupational activities performed in
the preceding 12 months through the use of an interviewer-administered Modifiable Activity Questionnaire (MAQ) (Kriska et al., 1990). “Sedentary individuals” reported no history of weight lifting, resistance training, or similar physical or occupational activities for a minimum of 6 months prior to enrollment in the study. “Concentrically-active individuals” were individuals who regularly performed concentrically-biased exercise training (e.g. road cycling, swimming) a minimum of 3 times per week for a minimum of 45 minutes each time at a moderate to high intensity for at least 12 weeks preceding study enrollment. Subjects were excluded if they had any known neuromuscular or immune diseases, recent injury or surgery to the lower extremities, or any contraindications to treadmill running. Subjects who passed the physical activity screening criteria and were included in the study were told to avoid any exercise or physical activities, the use of ice and/or anti-inflammatory medications, and therapeutic stretching of the lower-extremities both before and during the experimental period. Subjects were also instructed to maintain their normal habits and avoid starting any new supplement or medication regimens before or during the experimental period. Diet was controlled before and during the experimental period by asking subjects to maintain their normal eating habits, to refrain from altering their regular diet, and by scheduling the study visits at the same time of day for each subject. In total, 21 subjects were enrolled in the study and there were no dropouts or missing time points. “Concentrically active (CONC)” subjects were 22.5 ±4.4 year-old male and females (n=9; males=4, females=5). “Sedentary (SED)” subjects were 20 ±1.8 year-old males and females (n=12; males=4, females=8). There was no significant difference in height (CONC=172.9±8.2 cm, SED=170.2±10.8 cm, p=0.542), body weight (CONC=65.9±12.5, SED=60.9±12.1, p=0.372), or BMI (CONC=21.9±2.5, SED=20.8±2.2, p=0.321) between the groups of subjects. Not surprisingly, there was a significant difference between groups in average MET minutes/week
(calculated as the sum of leisurely and occupational activities reported on the MAQ for a full year preceding the experiment) between the groups (p<0.001), with the CONC subjects averaging 3342 ± 1404 MET min/week and the SED subjects averaging 698 ± 553 MET min/week. It should be noted that physical activity criteria for enrollment in the study was based upon the 6 months prior to enrollment in order to account for the repeated bout effect, while MET min/week values were calculated from MAQ data for 12 months prior to enrollment in the study, as the MAQ has been validated for a 12-month period.

**Experimental Protocol**

Following successful screening, subjects were scheduled for 5 separate study visits and the dependent measures were collected at 6 time points: Pre-exercise (Pre-Ex), Immediately Post-exercise (Post-Ex), 1 day post- (1 day), 2 days post- (2 days), 4 days post- (4 days), and 7 days post-exercise (7 days). These time points were specifically selected for several reasons. First, there is evidence to suggest that there may be a mechanistic role for MMP-9 at multiple time points following muscle damage (Sternlicht & Werb, 2001; Chen & Li, 2009), including the time points used in our study. In addition, this time course is well-characterized for other indirect markers of muscle damage (Clarkson & Hubal, 2002), and we have adopted this time course for our animal and human studies. All six visits were not separated by more than two hours during the day for a given subject. The first visit started with the collection of a pre-exercise blood sample using standard phlebotomy procedures. Next, rating of perceived muscle discomfort was assessed with a 0-100 mm Visual Analog Scale (VAS). Since discomfort is experienced across a range of muscles of the lower extremities after downhill running (Schwane & Armstrong, 1983) and the individual response is variable (Westerlind et al., 1992), subjects were asked to indicate
how much general discomfort they felt in their lower extremities while walking down a flight of stairs using preferred walking speed. The 100-mm, continuous visual analog discomfort scale was described to the subject with the following statement: “Please draw on ‘X’ on this scale to indicate how much discomfort you felt in your upper and lower leg muscles while you were walking down the stairs. The far left end of the scale represents the ‘least discomfort’ and the far right end of the scale represents the ‘most discomfort’ you have ever experienced in your muscles.” The subject was then asked to perform the concentric-only portion of a leg press task with both legs on a machine rigged with a pulley system allowing the researcher to perform the eccentric portion of the task. During this task, the hip joint extended from 100°-50° and the knee joint extended from 90°-5°. This task was repeated until a 1-repetition maximum (1-RM) was determined, which was defined as the maximum amount of weight a subject could lift in a single repetition. Multiple trials (but not more than 6) were used to determine 1-RM. Subjects were given 2 minutes of rest between trials. Subjects were asked to rate their perceived exertion (RPE) using the Borg exertion scale (Borg, 1982) following each trial, and the load was increased between trials according to the RPE, with larger increments added for lower RPE ratings and smaller increments added as RPE approached maximal effort. In an effort to minimize the influence of repeated maximal concentric contractions on the potential MMP-9 response, 1-RM trials were not measured in duplicate in this study. The test-retest reliability for our lab with a similarly administered 1-RM strength test measure from a previous study was 9.11-18.19 N (95% CI, CV=10.9%) (Madden et al., 2011).

Subjects were then prepared for the downhill running task. The downhill running was performed in a room with an ambient temperature that was between 20 and 23 degrees C. The specific downhill running protocol (steepness of angle [-10°] and duration of running bout [30
that we selected for our study is one that has been used by several other investigators, and has been associated with producing signs of muscle damage in humans (Byrnes et al., 1985; Sorichter et al., 1997; Koskinen et al., 2001a). In addition, this protocol most closely resembles the one used by Koskinen et al. (2001), but the duration of the run was reduced to ensure that sedentary subject could complete the task. A heart rate monitor (Polar WearLink™, Polar Electro Oy, Kempele, Finland) was positioned over the chest for determination of heart rate throughout the exercise. Age-predicted maximum heart rate (APMHR) was calculated using the equation: APMHR = 208-(0.7*Age) (Tanaka et al., 2001). Subjects were then allowed to warm up on the level (0°) treadmill for approximately 2 minutes and then speed was increased until a heart rate of 70% of APMHR was attained. This speed was maintained for a minimum of 3 minutes to ensure that a steady state of exercise was reached, without a change of heart rate from 70% APMHR. Then, the treadmill speed was kept fixed for the duration of the run but the grade was adjusted to a declined position of -10° (-17.45%) slope. Heart rate was measured throughout the exercise bout, but treadmill speed was kept fixed and not adjusted for any changes in heart rate that may have occurred. The subjects ran at -10° for 30 minutes. Ratings of perceived exertion using the Borg scale (Borg, 1982) were obtained at 5-minute intervals during the run. Following the run, subjects completed a 5-minute walking cool down. Immediately after the run, a post-exercise blood sample was obtained. Discomfort and 1-RM strength were also assessed. At each follow-up visit (1 day, 2 days, 4 days, and 7 days), a blood sample was collected and discomfort and 1-RM strength were re-assessed.

Blood sampling and storage

Blood was collected and stored as described previously (Madden et al., 2011) and in accordance with the pre-analytical recommendations for blood sampling to measure matrix
metalloproteinase levels (Jung et al., 2001b). Briefly, samples were drawn from an antecubital vein in the arm by venipuncture at each visit and collected in lithium heparin-coated vacutainer tubes (BD®, Franklin Lakes, NJ) then immediately centrifuged for 15 min at 1,500xg. Blood plasma was separated and immediately frozen and stored at -20°C in small volume aliquots until used for the assays described, while avoiding more than 3 freeze-thaw cycles for any given aliquot. All blood samples, pre- and post-exercise, were collected, handled, and stored under identical conditions.

**Plasma MMP-9 and TIMP-1 ELISA assay**

Plasma total MMP-9 and TIMP-1 were measured as described previously (Madden et al., 2011). Briefly, plasma samples were measured in duplicate by sandwich ELISA using commercially available kits (Quantikine, R&D Systems, Minneapolis, MN). Select samples were re-run in triplicate or quadruplicate because they were on the high- or low-end of the range of the standard curve and we wanted to ensure that any variability in sample concentration was a result of biological variation as opposed to measurement error. Samples were prepared per the kit recommendations with a 40-fold plasma dilution for MMP-9 and a 100-fold plasma dilution for TIMP-1 into Calibrator Diluent RD5-10 and RD5P, respectively, and measured using a microplate reader set to 450 nm with a correction of 540 nm. The intra-assay and inter-assay precision values provided for the MMP-9 kit are 2.3% and 7.5%, respectively. The expected range of values for heparinized plasma MMP-9 provided for this kit is 13.2 – 105 ng/mL. The intra-assay and inter-assay precision values provided for the TIMP-1 kit are 4.4% and 4.2%, respectively. The expected range of values for heparinized plasma TIMP-1 provided for this kit is 39 – 279 ng/mL. In a previous study (Madden et al., 2011), we determined the typical error of measurement (TEM) for the day-to-day (without any exercise) level of plasma MMP-9 to be
35.5 ng/ml (95% CI: 26.4-53.6 ng/ml) and for plasma TIMP-1 to be 13.72 ng/ml (95% CI: 13.0-23.7 ng/ml).

Creatine Kinase Activity Assay

Creatine kinase activity was determined as described previously (Madden et al., 2011). A commercially available kit (Diagnostic Chemicals Limited, Oxford, CT) was used for spectrophotometric analysis of creatine kinase activity with a peristaltic water pump maintaining a temperature of 37°C during analysis. Samples were prepared according to the kit and spectrophotometrically read at 340 nm for three minutes following a 2 minute pre-incubation period at 37°C. Activity per minute was recorded and translated into U/L using the equation provided with the kit. Each sample was quantified in duplicate. Samples that produced abnormally high or abnormally low values were re-run in triplicate or quadruplicate to verify sample concentration. Low and high control samples (DC-TROL level 1 & 2, Diagnostic Chemicals Limited, Oxford, CT) were also analyzed each day creatine kinases analyses were performed to verify that the assay was producing equivalent and expected results from day to day. The coefficient of variation for day-to-day precision for this kit is 4.0%, and within day precision is 2.3%. The expected ranges of CK levels provided in the kit are 20-184 U/L for males and 20-160 U/L for females when assayed at 37°C.

Statistics

Basic subject group demographics (height, weight, BMI, MET min/week) were compared using IBM SPSS Statistics version 19.0 (SPSS Inc., Chicago, IL, USA) with two-tailed, independent-samples t-tests and p<0.05 indicating a significant difference between groups. After examining the covariance structure of our time course data set, we decided that a repeated
measures ANOVA was not the most appropriate statistical model to use for this data because it would violate the homogeneity of variance assumption. Thus, we analyzed our time course data with a Linear Mixed Model approach assuming unstructured covariance in IBM SPSS Statistics version 19.0 (SPSS Inc., Chicago, IL, USA). In the time course comparisons between the SED and the CONC subjects, the fixed factors were Subject group and Visit. To examine trends over time within groups, independent linear mixed model analyses were used for the independent subject groups (CONC or SED) with Visit as the only fixed factor. Pre-Ex was used as the reference group for all analyses. To examine correlations between select dependent measures between or within groups, we examined Pearson product moment correlation coefficients with two-tailed tests of significance. P-values of less than 0.05 were considered significant. Data are presented as means ± SEM unless otherwise stated.

RESULTS

Both subject groups experienced a significant increase in discomfort over time (p<0.001), and specifically at the Post-Ex, 1 day, and 2 day time points following the downhill running task (Fig. 3-1a). There was no significant main effect for exercise group (p=0.85) and there was no interaction between exercise group and visit (p=0.090). CONC subjects experienced their peak discomfort at 1 day post (50.8 ± 6.8 mm), when discomfort was increased by 44 ±5.3 mm from Pre-Ex (6.8 ± 3.7 mm). SED subjects experienced their peak discomfort at 2 days post (43.3 ±7.9 mm), when discomfort was increased by 38.5 ±4.6 mm from Pre-Ex (4.8 ±1.2 mm). Subjects in both groups had no discomfort by 4- and 7-days post-exercise when compared to pre-exercise ratings (p=0.060 at 4-days and p=0.970 at 7-days).
Plasma CK activity was highly variable, but significantly increased by 162.9±177.2% in the pooled group of subjects at 1-day post-exercise (p=0.002), with no significant difference in the time course trend between the groups (p=0.813) (Fig. 3-1b).

Leg press strength was modestly but significantly decreased by 8.1 ±1.1% at Post-Ex and by 6.9 ± 1.6% at 1-day post-exercise in all subjects (Fig. 3-1c). There was no significant interaction between Exercise group and Visit (p=0.256), but leg press strength was only measured in a subset of the CONC subjects (n=4; males=2 and females=2), as this parameter was added to the study after it had already begun.
Figure 3-1. Traditional indicators of muscle damage across multiple time points following a downhill running task. White bars represent the concentrically-active (CONC) group and hatched bars represent the sedentary (SED) group. Visual analog scale soreness ratings (0-100 mm) after walking down a flight of stairs were increased from Pre-Ex at Post-Ex, 1 day, and 2 days with no temporal difference between the subject groups (a). Plasma creatine kinase (CK) activity (u/l) was significantly increased at 1 day post downhill running, although there was high subject variability (b). Leg press strength relative to Pre-Ex (%) was significantly decreased at Post-Ex and 1 day, and by 2 days there was only a trend toward a decrease in strength. By 4 days, strength was not different from Pre-Ex (c). Bars represent means ± SEM. *Denotes P<0.10. *Significantly different from Pre-Ex, P<0.05.
When all subjects were pooled and analyzed over time, plasma MMP-9 was modestly but significantly increased from 28.0 ± 1.9 ng/ml pre-exercise to 35.7 ± 2.3 ng/ml immediately post-exercise (p=0.009) and to 38.9 ± 5.3 ng/ml at 4 days post-exercise (p=0.017) (Fig. 3-2a). When Exercise Group was included as a factor, there was no significant interaction between Exercise group and Visit (p=0.132). When the groups were statistically examined independently using a linear mixed model approach with visit as the fixed factor, there was a significant increase in plasma MMP-9 at the Post-Ex time point in both groups (CONC p=0.004; SED p=0.019) and at 4-days post in the sedentary group only (p=0.041). Although there was one “high responder” at 4-days post-exercise, when this subject’s data was removed from the analysis, there was still a significant increase in plasma MMP-9 at Post-Ex (p=0.042) and 4 days post- (p=0.012) in the sedentary subjects. There was no obvious indication from the demographic data or the other dependent study measures in the study for why subject #20 had such a robust MMP-9 response (i.e., None of the indirect damage markers (discomfort, CK, or strength) were correlated to subject #20’s MMP-9 response). For plasma TIMP-1, there was no significant main effect for Visit (p=0.063), Exercise Group (p=0.136), or the interaction between Visit and Exercise Group (p=0.278) (Fig. 3-2b). The mean coefficient of variation (CV) for plasma MMP-9 and TIMP-1 activity measured between duplicates in the present study was 9.0% and 5.3%, respectively.
Figure 3-2. Plasma MMP-9 (a) and TIMP-1 (b) concentration (ng/ml) following a downhill running task. White bars represent the concentrically-active (CONC) group and hatched bars represent the sedentary (SED) group. When all subjects are pooled, plasma MMP-9 is significantly increased at Post-Ex and 4 days relative to Pre-Ex. Bars represent means ± SEM. *Significantly different from Pre-Ex, P<0.05
Changes in plasma MMP-9 at the Post-Ex and 4-days post-exercise time points, the sampling times when there was a modest but significant increase in plasma MMP-9, were not correlated with any other indicators of muscle damage (discomfort ratings, CK activity, and strength loss; correlations ranged from -0.208 to 0.324 with p-values all >0.10) when analyzed with all subjects pooled into one group (n=21). There were also no significant relationships between MMP-9 and muscle damage indicators when the analysis was performed within the two separate groups (CONC n=9; SED n=12; correlations ranged from -0.309 to 0.484 with p-values all >0.10). While strength loss was only measured in a subset of the CONC group (n=4), there was no significant correlation between MMP-9 levels and strength loss for the SED group only (n=12; Post-Ex p=0.739 and 4 days p=0.990), or for the pooled group of CONC and SED subjects (n=16) at the time points when MMP-9 was increased (Post-Ex p=0.656 and 4 days p=0.949).

DISCUSSION

The results from the study indicate that downhill running did result in indirect signs of muscle damage in both subject groups. Specifically, discomfort ratings and creatine kinase activity were both increased, and the magnitude and timing of the increase is similar to what others have reported using a downhill running model in humans (Schwane & Armstrong, 1983; Byrnes et al., 1985; Westerlind et al., 1992). Plasma MMP-9 was increased immediately post- and at 4-days post-exercise in sedentary subjects, but the increases were modest and variable across subjects. While the results from our present study support our hypothesis that plasma MMP-9 is increased after downhill running in subjects susceptible to lengthening-induced damage, these changes are small, within the day-to-day variability measured in a previous study (Madden et al., 2011), and do not correspond with other indirect indicators of muscle injury,
such as discomfort ratings, strength loss, or systemic CK activity. Thus, plasma MMP-9 and TIMP-1 levels do not appear to be clinically sensitive systemic markers of muscle damage following downhill running, a task that has been associated with muscle damage in both animals (Komulainen et al., 1994; Koskinen et al., 2001b) and humans (Schwane & Armstrong, 1983; Byrnes et al., 1985). Modification to the ECM by metalloproteinases may still be an important part of the sequence of events that characterize muscle damage and repair, but systemic levels of the ECM modifiers that were examined in this study did not appear to reflect the magnitude of damage that occurred following the task used and in the populations examined in this study.

Although a number of investigators have reported increased levels of circulating MMP-9 in clinical conditions that are characterized by increased muscle degradation such as rhabdomyolysis and myositis (Koskinen et al., 2001a), muscular dystrophy (Kieseier et al., 2001), amyotrophic lateral sclerosis (Demestre et al., 2005), and nerve crush injury (Demestre et al., 2005), the acute systemic response to a bout of exercise-induced damage is inconsistent in timing and magnitude (Koskinen et al., 2001a; Mackey et al., 2004; Madden et al., 2011). For example, a modest increase in serum MMP-9 activity has been reported following downhill treadmill running in physically-active individuals, but only in subjects who performed the task in a cold (5° C) room (Koskinen et al., 2001a). In a different study, systemic MMP-9 was increased at 8-days following an eccentric leg extensor task (Mackey et al., 2004), but there was no change immediately post-exercise, as was reported by our group and the Koskinen group (2001). In our previous study, we reported high systemic variability in plasma MMP-9 from day-to-day, and following an eccentric elbow flexor task in sedentary individuals, resulting in no significant increase in plasma MMP-9 at any of the time points examined within the first week of the exercise stimulus (Madden et al., 2011). In the present study, we do report a modest increase in
plasma MMP-9 immediately- and 4-days post downhill running, but the changes are less than those that we have reported from day-to-day in our previous work, and the individual response is still highly variable.

While the results of the present study are not entirely surprising to us based on the results from our previous work, we did implement a number of alterations to our study design to attempt to maximize the potential response. We modeled our study off of the only other human downhill running study examining systemic MMP-9 levels (Koskinen et al., 2001a), but with improved control of the subject population and blood sampling methods, and an increase in the number of subjects in the study. We did corroborate the immediately-post exercise increase in MMP-9 that the Koskinen group reported, but this change should be interpreted with caution given the variability in the plasma MMP-9 parameter that we have reported previously from day-to-day (Madden et al., 2011). Downhill running is a unique exercise stimulus that involves repeated eccentric contractions of the large muscles in the lower extremities. There are multiple aspects of this type of exercise stimulus that might explain why it produces a plasma MMP-9 response that is different from the response to an isolated muscle task. One aspect of downhill running that is different from an isolated muscle task is that it involves more muscle mass. If tissue volume is a component of the magnitude of the plasma MMP-9 response, then we expected the downhill running task to produce a more robust response since the task involved a substantial increase in active muscle tissue. The use of this type of whole-body exercise stimulus as opposed to an isolated muscle task may explain why we were able to detect a modest increase in plasma MMP-9 immediately- and 4-days post exercise in this study while we did not detect a response in the elbow flexor study, but the two exercise tasks are different in more ways than just the volume of tissue involved. Downhill running involves cyclical, high frequency contractions by multiple
muscle groups versus the lower frequency contractions that are performed by a single muscle or muscle group in other muscle damaging protocols, such as our elbow flexor model. In addition, the tissue volume argument has been refuted in the past for the creatine kinase response, as peak CK activity is reportedly lower for tasks involving a larger volume of muscle mass (i.e. downhill running) than for isolated muscle group eccentric tasks (Byrnes et al., 1985; Clarkson et al., 1992), but this issue has not yet been explored for the systemic MMP-9 response. There is some suggestion that while cyclical, high frequency eccentric contractions (i.e. downhill running) are adequate for producing muscle damage, the detectable indicators of muscle damage in the systemic circulation may follow a different time course than for high force, low frequency eccentric contractions (i.e. eccentric elbow flexor task). This is certainly true for systemic changes in CK activity, where downhill running typically produces a peak in CK activity at 12- to 24-hours post exercise (Byrnes et al., 1985), while isolated limb eccentric contractions produce a peak in CK activity at 4- to 5- days post exercise (Clarkson et al., 1992). Our study design included multiple post-exercise time points, allowing for the ability to detect a shift in the time course of the response from what has been reported previously, yet plasma MMP-9 levels only changed modestly at select time points and were the same as basal levels by 7-days post exercise, indicating that 1-week was an adequate amount of time to detect a response. However, it is possible that changes in MMP-9 and TIMP-1 levels occur rapidly and/or transiently in the blood circulation and without a higher blood sampling frequency through the use of microdialysis or an intravenous catheter, these changes could be missed. Very little is known or published about the efflux of metalloproteinases from the tissue to the bloodstream and the clearance from the bloodstream to the urinary tract, and a better understanding of this pathway may allow for better detection of metalloproteinases in the bloodstream.
Another way that we tried to maximize our likelihood of observing a systemic MMP-9 response was by selecting subject populations with a purported increased susceptibility to muscle damage. For our sedentary group, we recruited subjects who had not participated in any physical or occupational activities with their lower-extremities for a minimum of 6 months prior to the experiment in an attempt to control for the well-documented “repeated bout effect” (Byrnes et al., 1985), in which prior bouts of eccentric exercise reduce the magnitude of muscle soreness and damage in subsequent bouts. Based on years of work in this area, it is clear that the sedentary population has the greatest potential to experience muscle damage after an eccentric task (Byrnes et al., 1985; Clarkson & Hubal, 2002). If the magnitude of skeletal muscle damage is related to the magnitude of ECM remodeling that occurs, then the sedentary population should be the most likely to exhibit systemic changes in modifiers of the muscle ECM. The rationale for choosing the concentrically-active group came from a few studies that concluded that concentrically-biased exercise training results in changes to the sarcomere architecture that make sarcomeres more susceptible to damage (Ploutz-Snyder et al., 1998; Whitehead et al., 1998; Gleeson et al., 2003). Although this finding has been reported at least three times, the increased susceptibility in this population has also been questioned (Nosaka & Newton, 2002). In addition, the response of the muscle ECM to a potential change in the sarcomere architecture, or to concentrically-dominated training in general, is unknown, especially in the context of an eccentric exercise task. It is conceivable that concentrically-trained individuals may have a decreased drive for adaptation of the muscle ECM in response to eccentric exercise, as there may be more continuous adaptation present as a result of their training. There is some evidence that exercise training may produce more consistent ECM turnover and changes in collagen content (Mackey et al., 2004), either of which could make the ECM more resilient to eccentric exercise-induced
damage. Thus, selection of the concentrically-active subject population, while seemingly justified, may not have improved our chances of detecting a robust systemic MMP response, as the effects of concentric contractions on modifiers of the muscle ECM are unknown at this time.

Another issue regarding the association between exercise-induced muscle damage and MMPs is that several investigators have reported changes in MMP-9 protein and mRNA expression in muscle tissue and blood following exercise tasks that are not thought to be damaging. For example, Rullman and colleagues reported an increase in MMP-9 mRNA and protein from skeletal muscle extracts immediately- to 120 minutes after a single bout of a concentrically-dominated cycling exercise in recreationally-active individuals (Rullman et al., 2007). Urso and colleagues evaluated systemic MMP-9 levels in recreationally-active individuals before and after an 8-week long callisthenic training program (Urso et al., 2009). Although these subjects likely were protected from skeletal muscle damage as a result of their participation in the training program and the influence of the repeated bout effect (Byrnes et al., 1985), by the end of the training period, there was still an increase in serum MMP-9 immediately after they performed an acute resistance exercise test. These findings have lead to the question of whether or not remodeling of the extracellular matrix is unique to muscle damaging exercise, or associated with non-damaging exercise as well. It is not clear whether or not the type of exercise stimulus must be damaging to produce such a MMP response, and the systemic response does not appear to be reproducible or consistent at this time. Thus, although MMP-9 may clearly serve a mechanistic role in skeletal muscle damage and repair processes, the systemic circulation may not serve as the most reliable compartment for detecting these changes or for assessing the magnitude of damage.
In summary, data from the present study and our previous work suggests that circulating levels of MMP-9 and TIMP-1 do no reflect the magnitude of skeletal muscle damage or ECM remodeling that may be present after a single bout of downhill running. There is a substantial amount of variability in the systemic MMP-9 and TIMP-1 response in individuals who are sedentary and who perform regular concentrically-biased exercise training. These results suggest that alterations to the muscle ECM by metalloproteinases may only be detectable through more invasive techniques, such as muscle biopsies or microdialysis. In future studies, it may be valuable to expand the analysis of markers of muscle ECM remodeling beyond MMPs, and to compare ECM indices in multiple biological compartments. An understanding of the pathway of release and clearance of MMPs and other ECM modifiers may also be beneficial for maximizing the chances of detecting these indices. Finally, it is still unclear whether or not modifications to muscle ECM are unique to damaging contractions. A comparison of the effects of concentric versus eccentric contractions on the muscle ECM environment may provide some insight into the inconsistent timing and magnitude of the MMP response to a variety of damaging and non-damaging exercise paradigms that currently exists.
CHAPTER IV

INCREASED EXTRACELLULAR MATRIX DEGRADATION FOLLOWING ECCENTRIC VERSUS CONCENTRIC MUSCLE ACTIONS IN HUMANS

ABSTRACT

The matrix metalloproteinase-9 (MMP-9) response to a single bout of isolated eccentric or concentric muscle actions is unclear at this time. The purpose of this study was to determine if contraction type alters the ECM remodeling response in physically inactive humans. Subjects (n=21) were randomly assigned to 3 exercise groups: concentric-only (CON, n=7), eccentric-only on a free weight machine (ECC1, n=7), and eccentric-only on an isokinetic dynamometer (ECC2, n=7). Contraction type was isolated for each exercise group, but the task was generally the same and involved 6 sets of 10 maximal contractions of the non-dominant leg extensors. Functional (range of motion, leg circumference, soreness, strength) and blood (plasma creatine kinase, MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1)) parameters were measured prior to, immediately after, and 3-days after the exercise. After 3 days, a biopsy was collected from the Vastus lateralis of each leg with the unexercised leg serving as each subject’s control. Muscle was sectioned and stained for morphology, collagen type IV (COL4) and MMP-9 content, and homogenized to determine relative quantity of mRNA for 3 genes of interest: MMP-9, TIMP-1, and COL4. Plasma MMP-9 was increased and TIMP-1 was decreased immediately after exercise in the ECC2 group. Muscle MMP-9 was increased, and muscle COL4 content and TIMP-1 mRNA were decreased at 3-days post-exercise in the ECC2 group only. Interestingly, these indications of an ECM environment favoring degradation occurred in the absence of overt histological or functional signs of damage. Adaptations to the ECM are more apparent following eccentric than concentric muscle actions, and muscle damage may not be required to produce changes to the ECM environment surrounding skeletal muscle.
INTRODUCTION

Matrix metalloproteinase-9 (MMP-9) is a connective tissue-degrading enzyme that is capable of degrading the primary constituent of the basal lamina of skeletal muscle extracellular matrix (ECM), collagen type IV (Woessner, 1991; Sternlicht & Werb, 2001; Hyldahl & Hubal, 2014). There is a growing body of evidence to suggest that the timely activity of MMP-9 is crucial to successful muscle repair after damage (reviewed in (Sternlicht & Werb, 2001; Carmeli et al., 2004; Chen & Li, 2009; Hyldahl & Hubal, 2014)). The roles that have been proposed for MMP-9 in damage and repair events are generally associated with movement of molecules into or out of the matrix (Kessenbrock et al., 2010; Klein & Bischoff, 2011). Specifically, these processes may include degradation of ECM components to potentially assist with the migration and invasion of immune cells to the injured muscle fibers, liberation of necessary growth factors from the web of ECM that assist myogenic precursor cells in activation, proliferation, and differentiation, and liberation and migration of satellite cells during regeneration (Sternlicht & Werb, 2001; Carmeli et al., 2004; Chen & Li, 2009; Klein & Bischoff, 2011).

Increases in circulating levels of MMP-9 (serum or plasma) have been associated with a variety of types of exercise. However, the response (if existent) is small in magnitude (usually not greater than the day-to-day variation (Madden et al., 2011)), variable in timing across different exercise models (reviewed in (da Cunha Nascimento et al., 2014)), and not necessarily linked to whether or not the exercise was considered damaging to muscle. The typical fold change in circulating MMP-9 levels following various eccentric exercise models is only about 1.4-1.7-fold greater than resting levels (Mackey et al., 2004; Madden et al., 2011). The timing of the reported increases in MMP-9 varies and has been reported to happen immediately after exercise (Koskinen et al., 2001; Welsh et al., 2014), at 4 days post-exercise (Welsh et al., 2014),
and at 8-days post-exercise (Mackey et al., 2004), and it is unclear if the timing differences are related to the exercise model used in each study. It is also unclear whether or not the MMP-9 response in the blood is related to recent physical activity history or training status because an increase has been reported in both physically active (Koskinen et al., 2001) and physically inactive (Welsh et al., 2014) individuals. Finally, in several studies in which a prolonged training regimen was employed as the study intervention rather than a single acute bout of damaging contractions, increases in circulating MMP-9 have been reported either acutely after an exercise test or sometime throughout the course of the 8-week training period (Urso et al., 2009). These collective findings suggest that circulating levels of MMP-9 may not serve as a reliable or robust blood marker for acute muscle damage, but it is possible that the muscle MMP-9 response is more reflective of the adaptations that may be taking place in the tissue and surrounding ECM.

There are very few studies showing changes in muscle MMP-9 from human biopsy specimens following exercise (Mackey et al., 2004; Rullman et al., 2007; 2009), and even fewer reporting immunohistochemical data from stained tissue sections (Mackey et al., 2004; Rullman et al., 2009). The studies that do exist follow a similar equivocal response as the circulating changes for MMP-9, except the magnitude of changes in muscle MMP-9 mRNA or activity seem to be a little higher (2.3-3.0-fold (Rullman et al., 2007; 2009)) and the timing a little more consistent within exercise models (e.g., within the first few hours of completion of a cycling task (Rullman et al., 2007; 2009)). The muscle MMP-9 response to a single bout of isolated eccentric or concentric high-force muscle actions in subjects who are vulnerable to exercise-induced injury is unclear at this time, and has not been measured in conjunction with changes in circulating MMP-9, it’s inhibitor (tissue inhibitor of metalloproteinase-1 (TIMP-1)), or the content of collagen type IV (COL4) that is present in the ECM after the exercise task. Evidence supporting
a muscle ECM environment that favors degradation might include any combination of the following: increased MMP-9 mRNA or activity, decreased TIMP-1 mRNA or activity, or decreased COL4 mRNA or content in muscle. Changes in collagen remodeling markers have been reported following isolated concentric-only and eccentric-only contractions (Mackey et al., 2004), but the muscle MMP-9 response to isolated contraction types remains unclear at this time.

The purpose of this study was to determine if contraction type (concentric versus eccentric) alters the ECM remodeling response in physically inactive humans. Specifically, we examined muscle mRNA levels of MMP-9, TIMP-1, and COL4, muscle content of MMP-9 and COL4, and plasma levels of MMP-9 and TIMP-1, and compared these measures to other traditional indices of muscle damage following an acute bout of unilateral, isolated concentric-only or eccentric-only contractions while controlling for history of physical activity within the previous 6 months. We randomly assigned subjects to one of three groups; we used a single concentric (CON) model with a free weight system, and we used two different eccentric models: one with a free weight system (ECC1) and one with an isokinetic dynamometer (ECC2). The two eccentric models were included because the isokinetic dynamometer produced a more sustained stress throughout the range of motion than the free weight model as a result of failure midway through some repetitions. We wanted to include at least one eccentric group that would perform an amount of work that was matched to the concentric group to determine if the degree to which muscle is activated contributes to the response. With the failure partway through the task in the ECC1 group, we could not match the work performed with the CON group, so the ECC2 group was included as well. We hypothesized that circulating MMP-9 levels would be variable and not significantly increased following either type of exercise as we have previously seen in humans, but muscle MMP-9 levels, and other signs of ECM remodeling in the muscle, would be higher
with eccentric than concentric muscle actions because eccentric actions are more likely to provide a greater ECM remodeling stimulus than are concentric actions.

METHODS

Subjects

All subjects gave written, informed consent to participate in the study, which was approved by the University of Colorado (CU) Institutional Review Board and conducted in accordance with the 1964 Helsinki declaration and its later amendments. Subjects were 18-26 year-old (20.5±1.9 years), physically inactive males and females (n=21; males n=10, females n=11). Physical inactivity was defined as no participation in physical or occupational exercise or similar activities with the lower extremities for the previous 6 months as assessed by an interviewer-administered modifiable activity questionnaire (MAQ) (Kriska et al., 1990). Subjects were excluded if they were obese (BMI ≥30 kg/m²), pregnant (or amenorrheic for ≥35 days), allergic to topical lidocaine or other anesthetics, had any previous injuries or surgeries affecting the lower extremities, contraindications to performing lower extremity exercise, or if they had experienced any delayed-onset muscle soreness in the lower extremities within the previous 6 months.

Overview

Upon study enrollment, subjects were randomly assigned to an exercise group: concentric-only (CON; n=7), eccentric-only using free weights (ECC1; n=7), or eccentric-only using an isokinetic dynamometer (ECC2; n=7). The study included two experimental visits, with the first visit occurring at either the Applied Exercise Science Lab (CON and ECC1) or the Boulder Center for Sports Medicine (ECC2) (due to the location of equipment) and the second
visit occurring 3-days after the first visit at the CU-Boulder Clinical Translational Research Center (CTRC) with the support of the clinical staff for the biopsy sampling.

At the first visit, informed consent for the study was obtained and then the experimental procedures commenced. A venous blood sample was obtained prior to any measurements. Then, leg dominance was assessed using a simple, validated test (Hoffman, 1998). Functional leg measures (soreness ratings, leg circumference, range of motion (ROM), and maximal voluntary isometric contraction (MVIC) strength) were measured in each leg prior to the exercise task. The exercise task was only performed in the non-dominant leg. Immediately after the exercise task, a second blood sample was collected and the functional leg measures were repeated. Subjects were instructed to avoid any exercise or physical activities, the use of ice and/or anti-inflammatory medications, therapeutic stretching of the lower extremities, and to maintain normal dietary habits for the duration of the study. Subjects returned to the CTRC 3-days (±3 hours) following the start of visit 1. A venous blood sample was collected and the functional leg measures were repeated for each leg. A brief medical history and physical was performed by the CTRC physician to discontinue enrollment in any subjects who might have potential for an adverse response to the muscle biopsies, or if subjects had a medical history that would potentially interfere with the muscle damage and repair process (e.g., muscular disease or recent surgery). With the assistance of the researchers and nursing staff, the physician performed a muscle biopsy procedure to collect a single biopsy from the Vastus lateralis (VL) muscle in each leg, starting with the unexercised leg. Thus, the unexercised leg biopsy specimen served as each subject’s control specimen. Subjects were provided home-care instructions for their biopsy sites and discharged from the CTRC following the procedure. A schematic summarizing the study design is included as Figure 4-1.
Figure 4-1. This study was a randomized controlled trial with subjects randomly assigned to the concentric only (CON), eccentric-only on free weights (ECC1), or eccentric-only on an isokinetic dynamometer (ECC2) group following recruitment and successful screening. Besides exercise group assignment, all procedures were identical for the experiment. At visit 1, a blood sample was collected and functional measurements were made prior to the unilateral exercise task and this time point is referred to as Pre-Ex. Details about each exercise task can be found in the text of the methods section. Immediately following the exercise task, a second blood sample was collected and functional measures were repeated. Then, subjects returned to the lab for Visit 2 at 3-days post-exercise (3-days Post-Ex) so that a final blood sample could be collected, functional measures repeated, and biopsies collected from both the unexercised (control) and exercised (experimental) legs.
Measurement of Functional Parameter

Before (Pre-Ex), immediately after (Post-Ex), and 3-days after exercise (3 days Post-Ex), several functional parameters were measured in each leg. Muscle soreness was assessed by asking subjects to rate their perceived muscle discomfort for each leg using a 0-100 mm Visual Analog Scale (VAS) while seated at a bench immediately after performing 10 flexion-extension movements of the leg extensors for each leg throughout the range of motion (ROM). The 100-mm, continuous VAS was described to the subject with the following statement: “Please draw on ‘X’ on this scale to indicate how much discomfort you felt in your upper leg muscles while you were flexing and extending your leg. The far left end of the scale represents the ‘least discomfort’ and the far right end of the scale represents the ‘most discomfort’ you have ever experienced in your muscles.”

Knee joint range of motion (ROM) was measured with the subject laying prone on a massage-type table and by positioning the fulcrum of a manual goniometer on the lateral epicondyle of the femur and adjusting the goniometer arms to align with the greater trochanter of the femur and the lateral malleolus of the ankle while the subject fully extended and fully flexed his/her knee joint. Upper leg circumference was measured around the thigh at a location that was 20 cm proximal to the lateral epicondyle of the femur using a soft tape measure. These anatomical references were marked with permanent marker for identification at the second follow-up visit.

Unilateral maximal voluntary isometric contraction (MVIC) force was measured for each leg while subjects were seated on a custom built bench with their ankle secured with a soft strap to a steel cable affixed to a load cell. A directional pulley was used to redirect the angular torque
generated during the isometric contraction to the load cell. The knee joint angle used was 69.8±7.2° for the exercised leg and 69.4±7.0° for the unexercised leg (with full extension = 0°) for all subjects. MVIC was measured using a load cell and force transducer interfaced to an A-D converter (OMEGA Engineering, INC., Stamford, CT), and recording software (LabView, National Instruments, Austin, TX). Subjects performed 3 MVICs using each leg with 2 minutes of rest between trials and the force reported is the average of the 3 trials.

Exercise Task

The exercises tasks for this study involved contractions of the knee extensors in the non-dominant leg, with specific emphasis on the Vastus lateralis (VL) by careful subject positioning and monitoring during the exercise. The concentric (CON) group of subjects performed unilateral concentric-only leg extensions on a modified free weight leg extension machine using a load of 75% of each subject’s unilateral 1-repetition concentric maximum (1-RM). 1-RM was determined over no more than 6 single repetition trials with 2 minutes of rest between trials. The free weight machine was modified to include a rope pulley system attached to the moveable lever arm to which free weights were added. The pulley system allowed the researcher conducting the experiment to perform half of the work of the exercise (either lifting or lowering), depending on exercise group. Eccentric group 1 (ECC1) performed unilateral eccentric-only leg extensions (resisting flexion) using the same free weight machine described above, but with the researcher raising the weight with the pulley system prior to the start of each contraction. A load of 120% of each subject’s unilateral 1-repetition concentric maximum was used for the task, and 1-RM was determined as described for the CON group. Eccentric group 2 (ECC2) performed unilateral eccentric-only contractions on an isokinetic dynamometer set up to provide a maximal
force (220 ft.-lbf.) throughout the entire range of motion (ROM) during the exercise. Schematics of both pieces of equipment are provided in Figure 4-1.

For all exercise groups, subjects were seated at the apparatus with a hip joint angle (between the trunk and top of the thigh) of 101.5° ±6.8°. The waist and exercising leg were secured with soft straps and subjects were instructed to keep their hands on the apparatus grip handles near their hips during the exercise. Each exercise group performed the same number of sets and repetitions (6 sets of 10) for a total of 60 contractions, with identical rest periods between sets (2 minutes) and the same target contraction velocity (30 degrees/second). Contraction velocity was controlled for the CON and ECC1 groups by having subjects follow the speed of a custom-designed animation (Adobe Flash) representing movement of the limb through the ROM. For the ECC1 group, none of the subjects were able to maintain the lowering contraction velocity for all repetitions and subjects typically started failing (and dropping the load of free weights partway through the ROM) about halfway through the repetitions during set number 4, but they were instructed to continue working through the sets even after failure. For the ECC2 group, the contraction velocity was controlled by setting the isokinetic dynamometer to passive mode with a velocity of 30 degrees/second pushing down against the leg as the subject exerted maximal resistive force against a pad protecting the shin. The leg was returned to the fully extended position passively by the machine and subjects were reminded to relax during this phase.

The range of motion used for all 3 exercise tasks was across a knee joint angle range of as close to 85° as possible (starting angle of 90°, ending angle of 5°, with 0° equal to full extension) with some adjustments for subject comfort (±7.2°). Subjects were verbally encouraged throughout every contraction to continue exerting maximal effort, and they were periodically
reminded, as needed, to maintain appropriate positioning to maximize use of the VL. Subjects were asked to rate their perceived exertion following each set according to the Borg scale (6-20) (Borg, 1982) so that the researcher could generally gauge the effort put forth with each set.

**Blood Sampling and Analysis**

Blood was collected and stored as described previously (Madden et al., 2011) and in accordance with the pre-analytical recommendations for blood sampling to measure matrix metalloproteinase levels (Jung et al., 2001). Briefly, samples were drawn from an antecubital vein in the arm by venipuncture at each visit and collected in lithium heparin-coated vacutainer tubes (BD®, Franklin Lakes, NJ) then immediately centrifuged for 15 min at 1,500xg. Blood plasma was separated and immediately frozen and stored at -20°C in small volume aliquots until used for the assays described, while avoiding more than 3 freeze-thaw cycles for any given aliquot. All blood samples, pre- and post-exercise, were collected, handled, and stored under identical conditions.

Plasma total MMP-9 and TIMP-1 were measured as described previously (Madden et al., 2011). Briefly, plasma samples were measured in duplicate by sandwich ELISA using commercially available kits (Quantikine, R&D Systems, Minneapolis, MN). Select samples were re-run in triplicate or quadruplicate because they were on the high- or low-end of the range of the standard curve and we wanted to ensure that any variability in sample concentration was a result of biological variation as opposed to measurement error. Samples were prepared per the kit recommendations with a 40-fold plasma dilution for MMP-9 and a 100-fold plasma dilution for TIMP-1 into Calibrator Diluent RD5-10 and RD5P, respectively, and measured using a microplate reader set to 450 nm with a correction of 540 nm. The intra-assay and inter-assay
precision values provided for the MMP-9 kit are 2.3% and 7.5%, respectively. The expected range of values for heparinized plasma MMP-9 provided for this kit is 13.2 – 105 ng/mL. The intra-assay and inter-assay precision values provided for the TIMP-1 kit are 4.4% and 4.2%, respectively. The expected range of values for heparinized plasma TIMP-1 provided for this kit is 39 – 279 ng/mL. In a previous study (Madden et al., 2011), we determined the typical error of measurement (TEM) for the day-to-day (without any exercise) level of plasma MMP-9 to be 35.5 ng/ml (95% CI: 26.4-53.6 ng/ml) and for plasma TIMP-1 to be 13.72 ng/ml (95% CI: 13.0-23.7 ng/ml).

Creatine kinase activity was determined as described previously (Madden et al., 2011). A commercially available kit (Diagnostic Chemicals Limited, Oxford, CT) was used for spectrophotometric analysis of creatine kinase activity with a peristaltic water pump maintaining a temperature of 37°C during analysis. Samples were prepared according to the kit and spectrophotometrically read at 340 nm for three minutes following a 2 minute pre-incubation period at 37°C. Activity per minute was recorded and translated into U/L using the equation provided with the kit. Each sample was quantified in duplicate. Samples that produced abnormally high or abnormally low values were re-run in triplicate or quadruplicate to verify sample concentration. Low and high control samples (DC-TROL level 1 & 2, Diagnostic Chemicals Limited, Oxford, CT) were also analyzed each day creatine kinases analyses were performed to verify that the assay was producing equivalent and expected results from day to day. The coefficient of variation for day-to-day precision for this kit is 4.0%, and within day precision is 2.3%. The expected ranges of CK levels provided in the kit are 20-184 U/L for males and 20-160 U/L for females when assayed at 37°C.
Muscle Biopsy Sampling

A physician at the CTRC collected muscle biopsies during visit 2 (one from each leg) using the percutaneous needle biopsy approach with the assistance of the researchers and nursing staff. The sampling site in the mid-belly of the VL was first disinfected and then anesthetized with locally injected anesthetic (1% lidocaine hydrochloride injection, USP, without epinephrine, Hospira) prior to cutting a small incision (~1-2 cm) in the skin and a tiny nick in the fascia with a scalpel. The biopsy needle was inserted into the opening in the fascia to ensure proper needle placement in the muscle. A single, twisting and pushing maneuver was made with the needle trocar while under suction to extract the sample. Immediately after extraction, the sample was frozen in liquid nitrogen-cooled isopentane for 2 minutes, and then transferred into a cryotube and placed in dry ice until the sample reached its final storage location in a -80° C freezer. The subject’s biopsy incision site was then treated by closing the site with either steri-strips or a few biodegradable sutures and then bandaged with gauze and elastic wrap. A biopsy was first collected from the unexercised leg, and then from the exercised leg. Subjects were provided home care instructions for the biopsy sites and there were no adverse events associated with these procedures.

Muscle immunohistochemistry

Frozen sections (10 µm thick) were cut from the muscle biopsy specimens using a Leica 1900cm cryostat, placed on gelatin-coated slides, and stored at -20° C until use. For hemotoxylin and eosin (H&E) analysis muscle sections were stained to determine mononuclear cell number. Cells were counted using a marking feature in ImageJ (National Institutes of Health, USA) with
digital images that were collected with a 100x total magnification using a light microscope and Motic Images Plus 2.0 software.

For anti-type IV collagen and anti-MMP-9 immunohistochemical analyses, frozen muscle sections were thawed and air-dried at room temperature. Sections were incubated in blocking solution (3% BSA, 0.05% Tween-20, and 0.2% gelatin in phosphate buffered saline (PBS)) at room temperature for 1 hour. Samples were then incubated with the primary antibody solution (rabbit polyclonal to collagen type IV, Abcam, ab6586, 1:50; rabbit polyclonal to MMP-9, Abcam, ab38898, 1:100) at room temperature for 2 hours. Following incubation with primary antibody, slides were washed 3 times for 5 minutes with PBS, and then incubated with secondary antibody at room temperature for 1 hour. The secondary antibody solution was peroxidase-conjugated goat anti-rabbit IgG (H+L) (Vector) in blocking solution (1:50 dilution for collagen IV and 1:100 dilution for MMP-9). After three 5-minute washes in PBS, slides were developed with DAB substrate (prepared as directed by manufacturer) for 5 minutes at room temperature. Sections from each exercise group and from both control and exercised legs were always stained together to avoid large or systematic differences in staining intensity between the groups. Negative controls (all staining steps except primary antibody) were always included for each specimen for every staining batch.

Threshold analysis was used to determine collagen IV and MMP-9 staining quantity. Representative images were captured using a Moticam 5, 5.0 MP, microscope-mounted digital camera (Motic China Group Co.) and Motic Images 2.0 Plus software. Images of 4-8 fields were captured using a 10x lens objective (100x total magnification with ocular objective) for each muscle specimen. 255±74 fibers were analyzed for each specimen for the collagen IV analysis and 259±92 fibers were analyzed for each specimen for the MMP-9 analysis. Images were
opened using ImageJ, and converted into 8-bit grayscale photos. Threshold was adjusted for artifacts and ECM by adjusting the histogram of gray values to levels where red appeared only in the non-cell areas of the image, where collagen type IV and MMP-9 are thought to be present. Any void space areas in the images were subtracted off of the total pixel count prior to calculating a percent area. All images were manually corrected for obvious artifacts by subtracting artifact threshold pixels from the count. For the collagen IV analysis, two measures are presented: collagen type IV percent area and ECM percent area. ECM percent area was determined by inverting threshold images so that muscle fibers appeared red, and then adjusting the histogram so that red appeared only inside of muscle fibers in the image. With this method, ECM area is basically defined as all of the area that is not muscle fibers. This approach was taken because collagen IV is extremely abundant in the area between muscle fibers and we wanted to determine if all of the ECM area is stained by collagen IV using our IHC method (potentially as a result of conversion of a 3D tissue to a 2D image). Results for both collagen IV and ECM percent area are presented together.

*Muscle mRNA Analysis*

Muscle sections (~200 each 10 µm thick, ~20 mg tissue) were collected in an RNase-free cryotube while cutting cryosections, and kept at -20° C until the RNA isolation and purification procedure was performed. RNA was isolated using standard RNase precautions and by following the manufacturers instructions for the PureLink® RNA Mini kit (Life Technologies, USA) using silica-membrane column RNA isolation technology. Quantity and quality of RNA was determined by measuring the wavelength of the isolation elute at 260 and 280 nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Only samples with 260/280 ratios of 1.85-2.15 were used for downstream PCR applications. RNA concentration was adjusted to 10
ng/µl and 10 µl (100 ng total RNA) and used with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to make a 20 µl total volume single-stranded cDNA reaction using RT-PCR on a BioRad T100 thermal cycler (25°C for 10 min, 37°C for 120 min, 85°C for 5 min). Quantitative real-time PCR (qPCR) using iTaq Universal SYBR green Supermix technology (BioRad) was performed with the RT-PCR reaction products at a dilution of 1/10 (10 ng cDNA) for all samples (based on the optimized cDNA concentrations determined by running efficiency plates for each primer set). PrimeTime® qPCR Assays (Integrated DNA Technologies, USA) for three genes of interest (MMP9 [NM_004994], TIMP1 [NM_003254], the alpha-1 chain of type 4 collagen (COL4A1) [NM_001845]) and one reference gene (beta-actin (ACTB) [NM_001101]) were used in the qPCR reactions at a concentration of 500 nm for each forward and reverse primer. Annealing temperature was optimized for each primer by running temperature gradient plates with commercially-available control skeletal muscle RNA converted to cDNA with RT-PCR (Ambion AM7982, Life Technologies). For qPCR, samples were run in triplicate, intra-plate calibrators using the commercially-available control muscle cDNA were included with every plate to ensure plate-to-plate stability, and no template controls (NTCs) were included for each primer set. The optimized qPCR thermal cycling conditions for all 4 primers were the same: Initial template denaturation and enzyme activation at 95°C for 5 sec, [denaturation at 95°C for 5 sec, annealing/extension at 63.8°C for 30 sec] x 40 cycles, melt curve analysis temperature gradient from 65°C-95°C with 0.5°C increments at 3 sec/step. Quantification of mRNA for each gene of interest was normalized to the reference gene (ACTB) and was determined using the Pfaffl method for efficiency correction. Quantity of beta-actin normalized gene expression for each subject’s exercised leg was determined relative to gene
expression from each subject’s unexercised leg for the 3 genes of interest: MMP9, TIMP1, and COL4.

Statistical Analyses

Basic subject group demographics (age, height, weight, BMI) were compared using IBM SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA) and p<0.05 indicating a significant difference between groups. All changes in functional parameters are presented as a percent change from Pre-Ex to 3-days Post-Ex for the exercised leg only. Data from the unexercised leg was also collected for functional measures, but no functional parameters were changed in the unexercised leg (except MVIC) for any of the groups and this data is not included in the analysis. Blood parameters were analyzed using a linear mixed model approach in SPSS and assuming unstructured covariance with fixed factors including Exercise Group and Sampling Time. Pre-Ex was used as the reference group for all linear mixed model analyses. Muscle IHC and mRNA parameters were evaluated by determining the percent change between each subject’s exercised versus unexercised leg, and then differences between exercise group for each dependent measure were analyzed with a one-way ANOVA and Tukey HSD post-hoc analyses when there was a significant main effect. Graphs were created with SPSS v22.0. P-values of less than 0.05 were considered significant. Data are presented as means ± SEM unless otherwise stated.
RESULTS

Subject Characteristics

There were no significant differences in age (p=0.073), height (p=0.835), weight (p=0.970), or BMI (p=0.437) between the exercise groups, and each group included a mix of both male and female subjects. Subject characteristics are described in (Table 4-1).

Table 4-1 Subject characteristics for the three exercise groups are shown in the table. There were no differences in age, height, weight, or BMI across the groups. Standard deviation is shown in the table and differences were considered significant if p<0.05

<table>
<thead>
<tr>
<th>Exercise Group</th>
<th>n</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>7 (M=5, F=2)</td>
<td>21.0 ± 1.7</td>
<td>171.7 ± 10.3</td>
<td>69.1 ± 18.9</td>
<td>23.1 ± 4.5</td>
</tr>
<tr>
<td>ECC1</td>
<td>7 (M=3, F=4)</td>
<td>21.3 ± 2.3</td>
<td>172.9 ± 7.3</td>
<td>71.1 ± 13.5</td>
<td>23.6 ± 3.6</td>
</tr>
<tr>
<td>ECC2</td>
<td>7 (M=2, F=5)</td>
<td>19.1 ± 1.1</td>
<td>170.2 ± 6.9</td>
<td>70.3 ± 10.6</td>
<td>24.2 ± 2.6</td>
</tr>
</tbody>
</table>

Functional Parameters

There were no significant trends in most of the functional parameters measured over time (except MVIC) in the unexercised leg for any of the subject groups using a linear mixed model statistical test ((leg ROM p=0.067, leg CIRC p=0.322; soreness p=0.324), so changes in functional measures are only reported for the exercised leg, and they are relative to the pre-exercise time point. A summary of changes in the functional measures at the 3-day Post-Ex time point is provided in Table 4-2. To summarize, rating of muscle soreness in the exercised leg was significantly increased from 19.6 ± 18.8 mm at Pre-Ex to 36.9 ± 18.0 mm (on a 100 mm scale) for all groups immediately Post-Ex (p<0.001), but not at 3-days Post-Ex (p=0.480). There was a significant main effect for Sampling Time (p<0.001) but not for Exercise Group (p=0.417), indicating that all groups had a similar response. Exercised leg ROM was not changed for any
group at either post-exercise time point (Sampling Time \( p=0.081; \) Group \( p=0.103; \) average exercise ROM across all time points = 129.7 ± 8.0°), nor was leg circumference (Sampling Time \( p=0.425; \) Group \( p=0.946, \) average exercise leg circumference across all time points = 52.5 ± 5 mm). There was a small but significant decrease in MVIC strength immediately post-exercise compared to Pre-Ex in the ECC2 group only (-4.3 ± 10.55\%, \( p=0.023), \) but not in CON (6.1 ± 13.0\%, \( p=0.149) \) or ECC1 (-1.0 ± 14.1\%, \( p=0.379). \) There was a significant increase in MVIC strength in the exercised leg measured at 3-days Post-Ex relative to Pre-Ex (from 292.9 ± 94.5 N to 325.8 ± 95.9 N) in all groups (\( p<0.001 \)), but there was no main effect for Group (0.571).

MVIC strength was also increased at 3-days Post-Ex in the unexercised leg (\( p=0.041 \)) with no difference between the legs (\( p=0.653 \)) or across groups (\( p=0.785 \)) at this time point, suggesting that there may have been a learning effect associated with the MVIC strength measure in all groups.

Table 4-2 Changes in traditional/functional indicators of muscle damage for each exercise group are presented in table 4-2. Soreness and CK Activity are raw values from the 3-day Post-Ex time point, while the \( \Delta \) ROM, \( \Delta \) Leg Circumference, and \( \Delta \) MVIC values are expressed as a percentage change relative to Pre-Ex. One-way ANOVA analyses were performed to compare groups and differences were considered significant if \( p\leq0.05. \) There were no significant group effects for soreness (\( p=0.986), \) \( \Delta \) ROM (\( p=0.081), \) \( \Delta \) Leg Circumference (\( p=0.994), \) \( \Delta \) MVIC (\( p=0.224), \) or CK activity (\( p=0.884). \) Values are reported as means ± standard deviation

<table>
<thead>
<tr>
<th>Exercise Group</th>
<th>Soreness (mm)</th>
<th>( \Delta ) ROM (%)</th>
<th>( \Delta ) Leg Circumference (%)</th>
<th>( \Delta ) MVIC (%)</th>
<th>CK Activity (U/L) at 3-days Post-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>22.7 ± 15.1</td>
<td>-0.95 ± 3.5</td>
<td>0.10 ± 1.9</td>
<td>17.7 ± 11.0</td>
<td>107.4 ± 61.6</td>
</tr>
<tr>
<td>ECC1</td>
<td>21.4 ± 13.9</td>
<td>-1.0 ± 1.9</td>
<td>0.26 ± 2.7</td>
<td>6.4 ± 9.5</td>
<td>105.0 ± 51.8</td>
</tr>
<tr>
<td>ECC2</td>
<td>21.9 ± 14.1</td>
<td>-1.6 ± 3.9</td>
<td>0.16 ± 2.9</td>
<td>14.7 ± 15.3</td>
<td>142.8 ± 220.9</td>
</tr>
</tbody>
</table>
**Blood Parameters**

A linear mixed model statistical test was used to determine changes over Sampling Time and across Exercise Group for each of the blood parameters. Plasma CK was not significantly changed for either main effect following exercise (Sampling Time p=0.742; Group p=0.971), but there was an increase in the standard deviation in the ECC2 group at 3-days Post-Ex, suggesting that a few of the individuals did demonstrate an increase at this time point (Supplementary Figure 4-11).

Plasma MMP-9 was significantly increased from 48.4 ± 30.6 ng/ml at Pre-Ex to 127.4 ± 67.6 ng/ml immediately Post-Ex in the ECC2 group (p=0.002), with a significant main effect for Sampling Time (p=0.002), Group (p=0.002), and a significant Interaction (p<0.001), suggesting that there was not an increase in plasma MMP-9 in the CON or ECC1 groups, but only the ECC2 group (Figure 4-2).
There was a significant main effect for Sampling Time (p<0.001), a trend toward a Group effect (p=0.052), and a significant interaction (p=0.001) for plasma TIMP-1. The Sampling Time effect was most likely driven by the increased level of plasma TIMP-1 in the ECC2 group prior to exercise (Pre-Ex: CON=68.1±5.1 ng/ml, ECC1=65.1±7.9 ng/ml, ECC2=90.0±20.2 ng/ml). For the ECC2 group, plasma TIMP-1 decreased to levels that were not significantly different with CON and ECC1 at the Post-Ex (65.4±13.6 ng/ml) and 3-days Post-Ex (69.5±11.0 ng/ml) time points (Figure 4-3).
Figure 4-3 There was a significant main effect for Sampling Time \((p<0.001)\), a trend toward a Group effect \((p=0.052)\), and a significant interaction \((p=0.001)\). Pre-exercise basal plasma TIMP-1 concentration was significantly increased in the ECC2 group when compared to CON or ECC1 \((p<0.001)\), resulting in a relative significant decrease in plasma TIMP-1 in the ECC2 group at both post-exercise time points, even though the mean values at the post-exercise time points were not different between groups. Bars for each group are different shades (white=CON, patterned=ECC1, and black=ECC2) and represent means ± SEM. * indicates significantly different from Pre-Ex at \(p<0.05\)

Muscle Immunohistochemistry Parameters

There was not a significant difference in the number of mononuclear cells counted in H&E stained sections between exercise groups \((p=0.675)\) or between control or exercised legs \((p=0.434)\), nor was there a significant interaction between group or leg \((p=0.744)\). There were no overt signs of muscle damage (e.g., necrotic or swollen fibers) in any of the muscle biopsy specimens examined in the study.
Type IV collagen (COL4) staining in muscle resulted in an expected pattern of staining with COL4 localizing to the ECM (Figure 4-4). There was a significant main effect for Group for COL4 percent area (p=0.017) and ECM percent area (p=0.012) (relative to unexercised leg) in skeletal muscle biopsy specimens at 3-days Post-Ex. Percent change in muscle COL4 significantly decreased in both the ECC1 (-5.4±3.7%) and ECC2 (-9.3±3.2%) groups, but not in the CON group (4.2±2.1%) (Figure 4-5). There was a significant difference between CON and ECC1 (p=0.040) and CON and ECC2 (p=0.006), but not between ECC1 and ECC2 (0.370). Percent change in muscle ECM area followed the same trend and was decreased in both the ECC1 (-9.4±2.2%) and ECC2 (-10.4±4.1) groups, but not in the CON group (3.8±3.4%). There was a significant difference between CON and ECC1 (p=0.012) and CON and ECC2 (p=0.007), but not between ECC1 and ECC2 (0.835).

![Figure 4-4](image)

*Figure 4-4* Staining pattern for collagen type IV in muscle sections is localized to the ECM (A) and negative control staining showing lack of staining when COL4 primary antibody is excluded from staining procedure (B)
Figure 4-5 The percent area of collagen type IV (COL4) (white bars) is significantly decreased at 3-days post-exercise in the ECC1 and ECC2 groups compared to the CON group (p=0.017) with no difference between ECC1 and ECC2 (p=0.370). The same trend is present when using ECM percent area as a measurement tool (patterned bars). Bars represent means ± SEM. * indicates significantly different from unexercised leg at p<0.05.

MMP-9 staining in muscle also resulted in a pattern of staining with MMP-9 localizing to the ECM (Figure 4-6). There was a significant main effect for Group for percent change in area of MMP-9 (p=0.048) (relative to unexercised leg) in skeletal muscle biopsy specimens at 3-days Post-Ex. Percent change in muscle MMP-9 was significantly increased in ECC2 group (41.5±26.2%) when compared to the CON group (-14.0±14.4%), but the ECC2 group was not different from the ECC1 group (p=0.508), nor was the ECC1 group different than the CON group (p=0.294) (Figure 4-7).
Figure 4-6 Staining pattern for MMP-9 in muscle sections is localized to the ECM (A) and negative control staining showing a lack of staining when MMP-9 primary antibody is excluded from the staining procedure (B).

Figure 4-7 Muscle MMP-9 percent area of staining is significantly increased in ECC2 compared to CON (p=0.039), but there is no difference between CON and ECC1 (p=0.294) or between ECC1 and ECC2 (p=0.508). Bars represent means ± SEM. * indicates significantly different from unexercised leg at p<0.05.
**Muscle mRNA Parameters**

The normalized (to ACTB) relative quantity (relative to unexercised leg) of muscle COL4 mRNA was not significantly different between groups (p=0.464). There was at least one high responder in the ECC2 group producing very large error bars for this group and when this subject was removed from the analysis, there was a significant difference between groups (CON vs. ECC2, p=0.044; ECC1 vs. ECC2, p=0.048) with the greatest COL4 mRNA expression in the ECC2 group (Figure 4-8). The normalized relative quantity of muscle MMP9 mRNA was not significantly different between groups (p=0.802) (Figure 4-9). However, there was a significant decrease (p=0.005) in normalized relative quantity of muscle TIMP1 mRNA in the ECC2 group (0.60±0.07 NRQ) compared to both the CON (1.24±0.18 NRQ; p=0.037) and ECC1 groups (1.47±0.22; p=0.005) (Figure 4-10). There was not a significant difference between CON and ECC1 TIMP1 mRNA (p=0.601) expression in muscle.

![Figure 4-8](image)

**Figure 4-8** Normalized (to beta-actin) relative quantity (relative to unexercised leg) of muscle COL4 mRNA is not significantly different between groups when all subjects are included in the analysis (p=0.464), but when one high responder is removed from the ECC2 group, there is a significant difference between the ECC2 group and both the CON (p=0.044) and ECC1 groups (p=0.048). Bars represent means ± SEM. NS indicates not significant at p<0.05
Figure 4-9 Normalized (to beta-actin) relative quantity (relative to unexercised leg) of muscle MMP9 mRNA is not significantly different between groups (p=0.802). Bars represent means ± SEM.

Figure 4-10 Normalized (to beta-actin) relative quantity (relative to unexercised leg) of muscle TIMP-1 mRNA is significantly decreased in the ECC2 group compared to both the CON (p=0.037) and the ECC1 groups (p=0.005), but there is no difference between CON and ECC1 (p=0.601). Bars represent means ± SEM. * indicates significantly different at p<0.05.
There was not a significant main effect for Sampling Time (0.663) or Group (0.863) for plasma creatine kinase (CK) activity at a level of significance of $p \leq 0.05$. White bars represent the CON group, patterned bars represent the ECC1 group, and black bars represent the ECC2 group.

**DISCUSSION**

The main finding from this study is that maximal eccentric muscle actions sustained throughout the range of motion produce increased signs of ECM remodeling when compared to concentric muscle actions, even without the presence of overt histological or functional signs of muscle damage. Specifically, we showed that plasma MMP-9 was significantly increased immediately post-exercise and plasma TIMP-1 was decreased immediately post- and 3-days post-exercise in the ECC2 group. An increase in MMP-9 and a decrease in it’s inhibitor, TIMP-1,
would suggest increased activity of MMP-9 and an increased state of ECM degradation. In addition, at 3-days post-exercise, muscle collagen type IV and ECM percent areas were decreased, muscle MMP-9 staining was increased, and muscle TIMP-1 mRNA was decreased in subjects who performed eccentric muscle actions when compared to subjects who performed concentric muscle actions. These collective findings indicate that the ECM environment favored degradation to a greater degree in the group that performed sustained, maximal eccentric actions of the leg extensors when compared to concentric actions or eccentric actions using free weights sustained throughout less of the range of motion.

This is the first study examining changes in both plasma and muscle MMP-9 and other markers of ECM remodeling (collagen type IV and TIMP-1) in response to isolated muscle action types in a single leg with the opposite, unexercised leg serving as each subject’s control. In addition, screening criteria for study enrollment was strictly limited to physically inactive subjects who were unlikely to be protected from leg muscle soreness or damage through the repeated bout phenomenon. The findings from this study are consistent with what others have found in regards to signs of increased collagen remodeling in response to a single bout of eccentric exercise. Specifically, Crameri et al. found an increase in procollagen peptides indicating increased collagen breakdown within skeletal muscle in human subjects who performed a single bout of eccentric leg contractions (Crameri et al., 2004b). However, MMP-9 was not measured and there was only an eccentric exercise group included in the study. In another study, there was an increase in serum MMP-9 in response to a single bout of eccentric contractions of the knee extensors at 8-days post-exercise, but a change in collagen staining intensity was only found at 22 days post-exercise and the change was an increase relative to basal levels rather than an acute decrease that we report here (Mackey et al., 2004). In the
aforementioned study, there was not a concentric exercise group, so it is unclear whether or not the muscle action type used in the protocol was exclusively responsible for the changes reported. In the current study, we do not have data for changes to muscle at a time point that would be more consistent with the regenerative phase of muscle injury processes (between about 21 and 30 days post-exercise (Armstrong et al., 1983)), and it would be interesting to see if collagen IV content was altered at this time point, as reported by Mackey et al. (2004). In particular, it would be interesting in future work to know if isolated contraction type influences collagen content during a regenerative time point, even without the presence of overt histological or functional signs of muscle injury.

It is interesting that we report an increase in muscle MMP-9 content at 3-days post-exercise without a change in muscle MMP-9 mRNA following eccentric contractions. Meanwhile, a muscle TIMP-1 mRNA decrease was present at 3-days post-exercise. Work from our lab and others has suggested that the source of MMP-9 secretion may be independent of the muscle tissue and could be derived from prepackaged granular immune cells that dump their granules after extravasation into the muscle tissue in times of increased tissue remodeling (Mehan, 2013; Ardi et al., 2007; Li et al., 1999; Reihmane et al., 2012). Our muscle MMP-9 protein and mRNA results could be interpreted as consistent with this scenario. If the source of the increased MMP-9 content around muscle is coming from the circulating immune cells then it is possible that there may not be a drive for increased MMP-9 mRNA in homogenized muscle tissue because the source may not be from the tissue. Alternatively, it is possible that the 3-day post-exercise time point was not optimized for detection of an MMP-9 mRNA change, if existent.

We were somewhat surprised to not find overt histological signs of muscle damage in any of the biopsy specimens examined in this study, especially since the sampling time point we
chose is associated with the peak of the immune cell infiltration in rat (Armstrong et al., 1983) and human (Stupka et al., 2001; Beaton et al., 2002) damage models. It is possible that either we were unable to detect muscle damage in the small biopsy specimen collected or it did not occur. When collecting a biopsy specimen that is about half of the size of a pencil eraser from the Vastus lateralis muscle that is much larger than the sample, it is possible that damage may not be detected in the sample even though it might be present elsewhere in the muscle. In regards to the possibility of the absence of damage entirely, there are several other studies involving human muscle biopsy sampling that have reported no overt signs of muscle damage, yet changes in immune cell or satellite cell parameters (Pizza et al., 2002a; Crameri et al., 2004a). Specifically, there is evidence to suggest that both passive stretches and isometric contractions produce increases in neutrophils in muscle without overt signs of muscle damage (Pizza et al., 2002b). There is also evidence to suggest that immune cell activation is present in eccentrically-exercised skeletal muscle in the absence of any signs of lesions or damage to muscle fibers (Crameri et al., 2004a). Both immune cell infiltration and satellite cell activation may be regulated, in part, by the activity of MMP-9 (Sternlicht & Werb, 2001), so the possibility of changes to MMP-9, it’s inhibitor, TIMP-1, or its primary substrate, collagen type IV, without overt muscle damage seems reasonable. Given the lack of functional signs of muscle damage (changes in ROM, leg circumference, soreness, MVIC) in the present study, the latter possibility of a lack of muscle damage occurring in this study may be more likely. This is surprising since a single bout of unaccustomed maximal eccentric leg extension exercise has been shown to cause changes in functional and blood signs of muscle damage (MVIC strength, CK activity) with the number of contractions ranging from 30-300 (reviewed in Tiidus, 2008). It is possible that using 60 contractions was too few contractions to detect changes in functional measures, though the
number of contractions performed has not been able to explain the variability in damage measures previously reported (reviewed in Tiidus, 2008). Alternatively, the timing of our sampling at 3-days post-exercise could have caused us to miss the detection of changes that may have been present at a different time point.

What is interesting about the disparity in the MMP-9 response following exercise that is evident in the literature is that the studies reporting increases in MMP-9 do not always use exercise protocols that are considered to be damaging to muscle (reviewed in Clarkson & Hubal, 2002). Generally speaking, it is well established that eccentric contractions produce greater damage to muscle fibers than either concentric or isometric contractions (Newham et al., 1983; McCully & Faulkner, 1985), but the ECM and the MMP-9 response to concentric-only or eccentric-only contractions is unknown at this time. It is possible that the ECM adapts to multiple types of muscle actions without the presence of muscle damage, but there are very few studies isolating muscle action type and including muscle biopsies to answer this question. In the future, it would be interesting to translate this concept back to rodent models where it might be possible to have more control of the magnitude of muscle damage delivered to the muscle to see if there is a dose-dependent response in the ECM parameter changes that are reported here.

In summary, this is the first human study design incorporating a concentric-only exercise group in addition to eccentric-only exercise groups while measuring both circulating and muscle parameters related to ECM adaptations (MMP-9, COL4, TIMP-1). We report that adaptations to the ECM are the most apparent in subjects who perform sustained, maximal eccentric muscle actions, even without overt histological or functional signs of muscle damage present. Our study design was stronger than previous studies because we only included subjects who had not performed any physical activity or experienced any muscle soreness in the lower limbs for at
least 6 months prior to study participation. In addition, we had subjects perform the exercise with
only one leg so that the other leg could serve as each subject’s control, and we only collected
biopsy samples after the exercise task to avoid dealing with the damage that could be produced
from the biopsy sampling itself. In conclusion, adaptations to the ECM are more apparent
following eccentric than concentric muscle actions, and muscle damage may not be required to
produce changes to the ECM environment surrounding skeletal muscle in humans.
SUMMARY AND CONCLUSIONS

The purpose of this dissertation was to provide a more definitive examination of the plasma and skeletal muscle MMP-9 responses following exercise induced muscle injury in humans. Previous animal work from our lab and others had indicated that circulating or muscle MMP-9 was increased following eccentric exercise (Mehan, 2013; Koskinen et al., 2002) but these findings were inconsistent when translated to human models (Koskinen et al., 2001; Mackey et al., 2004; Rullman et al., 2007; 2009). I set out to improve upon study design decisions from previously published human work to maximize the potential of using damaging exercise as the stimulus while measuring MMP-9. Specifically, I controlled for recent participation in physical activity to avoid including subjects who were protected from damage as a result of the repeated bout effect. I also critically evaluated muscle force quantification approaches used in exercise-induced muscle damage experiments to determine if variability could be reduced depending on the measurement approach used. Unlike in previous studies, I added a test-retest measure to provide information on the stability of the dependent measures from day-to-day. I also looked across three different models of eccentric exercise since it wasn’t clear whether or not exercise model influenced the response. Finally, I selected one isolated muscle group exercise model to include biopsy sampling so that I could assess muscle measurements of MMP-9 and other indicators of ECM remodeling following exercise.

Prior to delving into the main focus of changes in MMP-9, I first tried to address a different issue regarding muscle force quantification following eccentric contractions. There was some suggestion that the optimal joint angle of muscle force production is shifted to longer muscle length following eccentric contractions and the degree of the shift could serve as a unique index for muscle injury that is more related to the magnitude of sarcomere damage than force
loss alone. Part of my motivation to pursue this diversion from the main MMP-9 aim of the dissertation was to determine if it was possible to reduce the variability associated with the measurement tools used in muscle soreness studies. If the approach to measuring force after eccentric exercise was producing an overestimation of the magnitude of force loss than this might explain some of the inconsistencies reported in previously published work. To evaluate force measurement approaches, I measured muscle force production following eccentric exercise of the elbow flexors using two methods: a fixed angle approach (90°) and an optimal angle approach (multiple angles throughout the range of motion). For the optimal angle approach, I measured force throughout a range of joint angles and then mathematically constructed force-angle curves to determine the optimal angle of force production (the vertex of the curve). For this experiment, I had physically-inactive subjects perform 6 sets of 10 lengthening contractions of the elbow flexors. I did find an optimal angle shift of about 10° to the right (longer muscle length) after eccentric exercise. However, when using an optimal angle approach to quantify force loss, the presence of an angle shift did not result in an over- or under-estimation of force loss when compared to using a fixed angle approach. Interestingly, in this experiment, I observed a “flattening” effect of the force-angle curves after eccentric exercise such that curve height was reduced by about 39% and not fully restored until 4-days post-exercise. At least one other published study has alluded to a similar change in force-angle curve characteristics following eccentric exercise (Butterfield & Herzog, 2005), but in my study, I proposed a method by which curve height changes could be quantified using information gained from the force-angle curve. I then examined whether or not the angle shift or curve height changes were related to other indicators of muscle damage to see if these curve characteristics could provide any unique information about the response to muscle damaging contractions. There were no correlations
between the angle shift or the change in curve height and other traditional indices of muscle damage. From this work, I concluded that additional experiments would be needed to determine the meaning of curve characteristic changes, but either a fixed angle or optimal angle measurement approach is adequate in quantifying force loss following eccentric contractions. For future research involving isolated muscle exercise tasks, I made the suggestion to use a fixed angle force measurement approach since it provides similar information to the optimal angle approach but is less time consuming.

Next, I returned to the main MMP-9 focus of the dissertation and examined plasma changes in MMP-9 before and at several time points after the elbow flexor protocol described in the paragraph above. I tried to maximize my chances of causing damage to the muscle by including only physically-inactive subjects, using high-force eccentric contractions of the upper extremity muscles that are less likely to be protected via the repeated bout effect, and including only male subjects to prevent the potential influence of the menstrual cycle on the dependent measures. I also included a second pre-exercise time point to assess the stability of the dependent measures, particularly plasma MMP-9, since this had not been included in previously published work. I found that there was no significant change in either plasma MMP-9 or its inhibitor, TIMP-1, at any of the post-exercise time points, and the day-to-day variability in plasma MMP-9 exceeded the significant increases that others had reported in previously published human work. These findings led me to consider using a different model of eccentric exercise involving a larger muscle mass to address the possibility that tissue volume involved in the exercise might be related to the magnitude of the circulating MMP-9 response. From these findings, I decided to use a downhill running model for the next study to increase the total tissue volume involved in the exercise and to create a more consistent human model parallel to the mouse model that we
had already used in our lab with reported changes in circulating MMP-9 in WT mice (Mehan, 2013).

For the next study, I included two subject groups with purportedly increased susceptibility to lengthening-induced muscle injury: sedentary individuals and individuals who performed concentrically-biased exercise training. Subjects ran downhill for 30 minutes and plasma MMP-9, TIMP-1, and other traditional markers of muscle damage were measured before and at several time points after the exercise task. I found that plasma MMP-9 was significantly increased immediately post-exercise in both subject groups, and at 4-days post-exercise in the sedentary group only. The magnitude of the MMP-9 increase was smaller than the day-to-day variability that we had reported in the previous experiment, and was variable between- and within-subject groups. There were no correlations between plasma MMP-9 changes and other traditional indices of skeletal muscle damage. From this work, I concluded that plasma MMP-9 changes may be detectable in the systemic circulation after downhill running, but changes are small and do not correspond to other markers of damage. The modest MMP-9 increase that we reported in this study after using a larger muscle mass than the previous exercise model did generate interest in the idea that MMP-9 may still serve an important role inside of muscle tissue. Animal findings from our lab (Mehan, 2013) and others (Koskinen et al., 2002) corroborated the idea that changes in muscle MMP-9 and other markers of ECM remodeling were important to pursue in the human model. The difference in the response between the concentrically-active and sedentary subjects in this study also raised the possibility that concentric training, or concentric muscle actions in general, may provide a unique stimulus to the ECM. It is conceivable that concentrically-trained individuals may have a decreased drive for adaptation of the muscle ECM in response to eccentric exercise, as there may be more continuous adaptation present as a result
of their training. The response of the ECM to isolated concentric or eccentric muscle actions was unclear, and I planned to incorporate this question into the next experiment.

Thus, for the final experiment in this dissertation, I chose a leg extensor exercise model that would be safe and appropriate for biopsy sampling from an isolated muscle, the *Vastus lateralis*. Physically inactive subjects performed 6 sets of 10 either concentric-only or eccentric-only muscle actions with their non-dominant leg only so that I could determine if contraction type (concentric versus eccentric) alters the ECM remodeling response. A biopsy specimen was collected from each leg (exercised and unexercised) at 3-days post-exercise and the unexercised specimen served as each subject’s control. An additional motivation for becoming interested in the role of contraction type on the plasma or muscle MMP-9 response was that in the time between the downhill running study and this leg extensor study, a group of investigators published a series of experiments showing changes in MMP-9 and other markers of ECM remodeling following concentrically-biased cycling exercise (Rullman *et al.*, 2009; 2012). The cycling model that was used in their work is not typically considered to be damaging to muscle, and I became interested in the idea that ECM modification may be present and important even in the absence of contractions that are damaging. In this leg extension study, I found that plasma MMP-9 was increased and TIMP-1 was decreased immediately post-exercise in the group that performed sustained, maximal eccentric contractions when compared to the concentric group, even though overt histological and functional signs of muscle damage were absent in all subject groups. In addition, muscle MMP-9 content was increased, while muscle COL4 content and TIMP-1 mRNA were both decreased at 3-days post-exercise in the eccentric group, but not in the concentric group. These collective results suggest that maximal, sustained eccentric muscle
actions produce increased signs of ECM remodeling when compared to concentric muscle actions, even without the presence of overt histological or functional signs of muscle damage.

Collectively, the results from the series of studies that are presented in this dissertation suggest that there is an immediate post-exercise increase in plasma MMP-9 in humans after performance of eccentric muscle actions, but the response is variable across subjects and small in magnitude. ECM adaptations that are detectable inside of muscle are more prevalent after eccentric muscle actions compared to concentric muscle actions, and muscle damage may not be required to stimulate modification of the ECM.

While the thought of discovering a non-invasive blood marker of diagnostic value that changed in proportion to the magnitude of damage that occurs was seemingly justified and exciting at the onset of this dissertation, the results from our experiments would suggest that plasma levels of MMP-9 are not suited for this purpose. This conclusion does not preclude an important role for MMP-9 in the cellular damage and repair processes that occur inside of muscle, and, in fact, our muscle data supports an active role for MMP-9 in the tissue following eccentric muscle actions. Specifically, the early rise in plasma MMP-9 and the changes to the ECM that are present in the days following eccentric muscle actions support one of the proposed mechanistic roles for MMP-9 in assistance with the migration and invasion of immune cells to muscle tissue. Alternatively, it is possible that MMP-9 may be responsible for liberating growth factors or other important signaling molecules from the web of ECM proteins after an eccentric stimulus so that adaptations of the ECM can occur in preparation for future muscle actions. Several mechanistic roles have been proposed for MMP-9 in muscle tissue and ECM remodeling, and while the timing of the MMP-9 changes in our data is consistent with some of the proposed
roles, more work needs to be done to characterize the specific role for MMP-9 in human muscle damage and repair processes.

Our work has contributed several new findings to this field that continue to be cited by others. For example, in our elbow flexor study, we challenged the idea that changes in plasma levels of MMP-9 following exercise are real or meaningful, as others had suggested, because of the high day-to-day variation that we were the first to report. We also identified several potential confounds in previously published work and attempted to resolve those deficiencies in our own study designs to reduce the potential variability of the MMP-9 response, but we still found inconsistent plasma changes in MMP-9. In the final experiment in this series we were the first to add MMP-9 and TIMP-1 measures to a study that was designed to isolate the effects of contraction type on measures of ECM remodeling and our findings support a role for MMP-9 in ECM remodeling of human muscle exposed to an eccentric stimulus.

In future work to clarify the role of MMP-9 in exercise induced skeletal muscle injury in humans, it would be more appropriate to focus efforts on changes that are happening inside of the tissue, rather than the circulation. In addition, our work suggests that the stimulus for ECM remodeling may not need to be as extreme as was once thought, as we found changes in the ECM after a single bout of exercise without any signs of muscle damage. Future work focusing on human muscle tissue should also include an expanded sampling timeline so that it is less likely that changes will be missed as a result of not enough sampling time points. Designing a study involving muscle biopsy sampling with an increase in the number of samples collected is extremely challenging in intact humans because the biopsy procedure itself can cause damage to the muscle tissue. An alternative approach might be to return to a rodent model for this type of study to gather more specific information about the timing of the MMP-9 or ECM remodeling
response, and then return to a human model with a more solid justification for the time points selected for the experiment.

It is tempting to speculate that MMP-9 or TIMP-1 might become important targets for drug development for clinical conditions characterized by muscle or ECM damage, and our work would support this venture. MMPs and TIMPs have certainly gained attention as potential drug targets for reducing the ability of tumors to migrate through the ECM. There is less information on the feasibility or utility in designing a MMP/TIMP-based drug that would be specific to the ECM surrounding muscle and this area of research seems to have merit. On a more practical level, understanding more about the role of MMP-9 in muscle and ECM remodeling in humans could be beneficial to identifying how to tailor exercise-based rehabilitation to facilitate ECM adaptations following exercise induced muscle injury. While the work discussed in this dissertation supports a role for MMP-9 in exercise induced muscle injury in humans, many questions remain to be answered in this field.
REFERENCES


APPENDIX A

Abbreviations

1-RM: 1-repetition maximum
ACSM: American College of Sports Medicine
APMHR: age-predicted maximum heart rate
BMI: body mass index
CK: creatine kinase
COL4: collagen type IV
CON: concentric-only exercise group (Ch. 4)
CONC: concentrically-active subject group (Ch. 3)
CTRC: clinical translational research center
CV: coefficient of variation
DHR: downhill run
DOMS: delayed onset muscle soreness
ECC1: eccentric-only exercise group on weight machine (Ch. 4)
ECC2: eccentric-only exercise group on isokinetic dynamometer (Ch. 4)
ECM: extracellular matrix
EIMD: exercise-induced muscle damage
ELISA: enzyme linked immunosorbent assay
IP: immediately post-exercise
MAQ: modifiable activity questionnaire
MET: metabolic equivalent of task
MMP-9: matrix metalloproteinase-9
mRNA: messenger RNA
MVIC: maximal voluntary isometric contraction
Pre1: Pre-exercise time point 1
Pre2: Pre-exercise time point 2
PreEx: Pre-exercise
qPCR: quantitative real-time polymerase chain reaction
ROM: range of motion
RPE: rate of perceived exertion
RT-PCR: reverse transcription polymerase chain reaction
SED: sedentary subject group (Ch. 3)
SEM: standard error of measurement
TEM: typical error of measurement
TIMP-1: tissue inhibitor of metalloproteinase-1
VAS: visual analog scale
VL: Vastus lateralis
WT: wild type
APPENDIX B

Additional photos and schematics from elbow flexor apparatus (Chapters 1 and 2 of dissertation)

Figure B-1. For the elbow flexor study (Chapters 1 and 2), we constructed a custom apparatus to use for the muscle force-joint angle measurements and the eccentric exercise task. For both tasks, subjects were seated on a stool at the structure made from SuperStrut® material. The non-dominant arm was supinated and supported at 90° of shoulder flexion in the sagittal plane by a padded platform. Maximal voluntary isometric strength at multiple angles of elbow flexion throughout the range of motion was measured using a force transducer interfaced to an A-D converter recording software. The load cell for the force transducer was positioned at a 90° angle to a fixed lever that transmitted the torque from the isometric contraction, as shown in image B-1a above. The angle for each trial was selected using a fixed angle wheel with pin holes approximately 10° apart so that about 11 total angles could be measured for each subject. For the eccentric task, the same structural apparatus was used, but without the fixed angle wheel or force transducer. Instead, the subject held onto a handle attached to a wire cable. The wire cable was directed through a pulley and weights were attached to the end of the cable, as shown in image B-1b.

Figure B-2. Close up image of the force-angle mechanism of the apparatus. A) force transducer, B) fixed angle wheel, i) stationary lever that attaches the force transducer to measure the isometric force, ii) breaking device, iii) slotted wheel, C) arm lever, D) padding, E) lower lever, F) one of the angles at which isometric force is measured.
APPENDIX C

Data from an experiment on the diurnal variation in plasma MMP-9

Research question: What is the influence of time of day on plasma MMP-9 levels in humans throughout the hours of the day when sampling will be conducted in our experiments?

Hypothesis: There is no difference in plasma MMP-9 levels in humans across different times of the working day.

Methods: Venous blood was collected from 6 subjects in the lab at 3 different time points throughout the day that correspond to times when our experiments are typically conducted (0800, 1200, and 1630). Subjects were instructed to maintain their normal dietary, physical activity, and sleep habits prior to and during the experiment. Venous blood was collected in a heparinized vacutainer and immediately centrifuged for 15 min at 1500 rpm to separate the plasma. Plasma was stored on dry ice for transport to the -20 degree freezer, and then remained in the freezer until the analysis was performed. Plasma MMP-9 levels were determined using standard gelatin zymography procedures that are described elsewhere (Madden et. al. 2011) and samples were loaded in zymogram lanes with an equal protein content as assessed by a Bradford assay. A linear mixed model analysis in SPSS was used to analyze the data with p<0.05 for significance.

Results: There was no statistically significant difference in plasma MMP-9 integrated density across time of day for the times tested in this experiment (p=0.190).

Conclusions: For future experiments, venous blood samples can be collected at various times throughout the day without concern of a potential diurnal influence for plasma MMP-9 levels.