# UTILIZATION OF MILITARY-RELEVANT MUSCLE INJURY MODELS TO IDENTIFY PHARMACOLOGICAL TREATMENT STRATEGIES

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## ABSTRACT

This study was designed to characterize critical molecules involved in skeletal muscle response to ischemia-reperfusion (I/R) and blunt trauma injury in order to identify strategies for pharmacological intervention. For I/R injury, a tourniquet was applied to rat hind limbs for 3 h. Extensor digitorum longus (EDL) muscles from the healthy and I/R leg were harvested 2 h post-reperfusion for analysis. For the blunt trauma model, mice were anesthetized, the tibialis anterior (TA) muscle was exposed, and injury was induced by applying a steel probe (cooled to -20 C) to the belly of the TA muscle. Muscle samples were collected from the healthy and injured leg 3, 10, 24, 48, and 72 h post-injury. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR), immunoblotting, and immunohistochemistry were used to quantify/localize analytes of interest. I/R resulted in a significant ($p<0.05$) upregulation of MT (4.7-fold) and MMP-9 (2.6- fold) mRNA, with no change in MMP-2. Blunt trauma/freeze injury resulted in an upregulation of mRNA expression at 3h, 10h, 24h, 48h, and 72h, respectively for MT (2.15-, 3.43-, 4.23-, 3.50-, 1.2 (n.s.)- fold), MMP-2 (1.25-(n.s), 1.28-(n.s), 1.07-(n.s), 1.84- and 1.86-fold), and MMP-3 (4.70-, 67.68-, 40.04-, 24.29, and 14.0-fold). Protein levels of MT and MMP-2 also increased several-fold post-injury with a decrease in the active form of MMP-3. We conclude that MT production is related to injury-induced oxidative-stress, which affects extracellular matrix integrity, explaining our observed increase in the MMPs. Thus, oxidative-mediated extracellular matrix disruption is a potential mechanism for skeletal muscle proteolysis post-injury.

## 1. INTRODUCTION

Today's Warfighter is subject to a wide variety of traumatic injuries to skeletal muscle and surrounding tissues. Tourniquet use for hemorrhage control is common in military trauma, as well as in surgical situations requiring the restriction of blood flow to an extremity. Often, the subsequent reperfusion of blood to ischemic tissue induces an acute inflammatory response. This response can exacerbate a pre-existing injury in skeletal muscle, making ischemia/reperfusion (I/R) injury a concern in military personnel.

In regards to acute injury, one of the most common injuries is blunt force trauma due to improvised explosive devices (I.E.Ds). Blast injuries have become common in military conflicts. As of December 2006, 63% of injuries sustained in combat are the result of explosive munitions including bombs, grenades, land mines, missiles, and mortar/artillery shells (Spotswood 2006). The lifetime cost of providing treatment, disability payments and healthcare to war veterans sustaining such injuries will likely range between $300 and $600 billion, depending on how long the war lasts (Bilmes 2008).

Skeletal muscle tissue in the limb sustaining blast injury and subsequent I/R is affected, in some cases resulting in the loss of function and incomplete rehabilitation. Prolonged hospitalization and subsequent rehabilitation impact the number of active duty and reserve personnel available for combat/military missions. Understanding the critical signaling cascades induced following military-relevant injuries of this nature would help to identify potential new pharmacological target sites to minimize the extent of injury while attenuating time to recovery.

### 1.1 Skeletal Muscle Injury

The healing process of a skeletal muscle injury begins with an inflammatory response which is initiated within hours of the injury and can last for three to five days. This response is critical to skeletal muscle repair. During the inflammatory reaction the body produces

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compounds and cells which remove dead skeletal muscle fibers and start the repair process (Jarvinen et al. 2005). In some cases, when the injury is severe, the inflammatory reaction is exaggerated and the result is further injury and delayed healing (Sicard 2002). This may be due to an excessive release of factors that initiate the inflammatory response, but prevent the release of necessary factors involved in the repair process. This is problematic because muscle repair relies on an intricate, finely orchestrated process. While it is evident that skeletal muscle regeneration involves changes in the synthesis and degradation of extracellular matrix components, the molecular adaptations in skeletal muscle affecting function and recovery following traumatic injury are not currently known.

### 1.2 The Role of Matrix Metalloproteases (MMPs) and Metallothioneins (MTs)

Concomitant expression of metallothioneins (MTs) and metalloproteases (MMPs) have been documented in skeletal muscle that has sustained an injury or is undergoing atrophy (Jagoe et al. 2002; Lecker et al. 2004; Urso et al. 2007; Warren et al. 2007). MTs are small (12-14 kDa), ubiquitous, cysteine-rich, zinc-binding proteins which are primarily produced in the liver and released into the circulation (Tapiero; Tew 2003). Upon release into the circulation the protein plays a pivotal role in cellular processes to render protection to all tissues of the body. In skeletal muscle, MTs initiate anti-inflammatory and anti-apoptotic signaling cascades, inhibit against reactive oxygen species (ROS)-induced cytotoxicity, protect against ROS-induced DNA degradation and maintain zinc homeostasis (Feng et al. 2006; Miles et al. 2000; Tapiero; Tew 2003). Marked induction of MT mRNA is evident in skeletal muscle of animals and humans in conditions that promote decreased protein synthesis and increased protein degradation, including injury and disuse (Jagoe et al. 2002; Kondo et al. 1994; Lecker et al. 2004).

The MMP family of enzymes contributes to both normal and pathological tissue remodeling. MMPs act as regulatory molecules, both by functioning in enzyme cascades and by processing matrix proteins, cytokines and growth factors. Optimal remodeling of the ECM is contingent on tightly regulated MMP activity in a location-, time-, and concentration-dependent manner (Kjaer 2004). Certain stimuli, including MTs, activate MMPs subsequently influencing extracellular matrix production and degradation (Giannelli et al. 2005; Kjaer 2004). Evidence exists to suggest that MMP-2, MMP-3, and MMP-9 are responsible for anti-inflammatory processes and collagen remodeling in skeletal muscle (Kjaer 2004; Nagase et al. 2006). However, there are few data characterizing the effects of skeletal muscle injury on the various MMP isoforms at both the mRNA and protein level.

Recent work has shown a concomitant increase in MT and MMP mRNA expression during skeletal muscle remodeling (Jagoe et al. 2002; Urso et al. 2006). Several lines of evidence suggest that there is involvement of oxidative stress in the cascade of events initiating skeletal muscle remodeling, particularly following injury when skeletal muscle cells are more susceptible to oxidative stress (Jagoe et al. 2002; Lecker et al. 2004; Warren et al. 2007). These findings imply that the signaling cascade that connects injury, the release of ROS, MT induction, and MMP-induced remodeling is a prime candidate for pharmacological intervention.

### 1.3 Purpose of Study

The purpose of this study was to determine the effects of two specific military-relevant injuries (I/R and blunt force trauma) on the expression and activity of MT, MMP-2, MMP-3 and MMP-9 in skeletal muscle. We first explored the acute effects of I/R at 2 h post-injury to obtain a snapshot of those changes that occur within the first few hours post-injury. Following analysis of those data, we then characterized the effects of a more traumatic (blunt force/freeze) injury on the expression of these molecules from 3 to 72 h post-injury. It was hypothesized that there would be an acute increase in MT mRNA and protein products, with a subsequent increase in MMPs at later time points, specifically during the remodeling phase (e.g. 24- 72 h post-injury). We also hypothesized that there would be a concomitant increase in MT and MMP proteins within the extracellular matrix using immunohistochemistry, characterizing the role of MT and MMPs in orchestrating extracellular matrix protection and remodeling, respectively.

## 2. METHODS

We employed two models of military relevant injury: ischemia/reperfusion injury in rat extensor digitorum longus (EDL) muscle and blunt/freeze trauma injury in mouse tibialis anterior (TA) muscle. Muscle samples collected from healthy and injured limbs were assessed for MT, MMP-2, MMP-3, and MMP-9 content at both the mRNA and protein level as described below. All animals were housed and cared for in accordance with the Guide to the Care and Use of Laboratory Animals in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). After arriving at the respective facility, animals were given 14 days to acclimate to their housing prior to the initiation of experimentation. Daily measurements of body weight, food intake and water intake were made to ensure the health of the animals

throughout experimentation. Animals were housed on a 12 h light/dark cycle, at ambient temperature (30 ± 1 ºC). Animals were provided with food and water ad libitum prior to and following all procedures.

All procedures were performed under 1.5-2.5 % isoflurane anesthesia in oxygen, administered via nosecone. Body temperature was maintained at 37 ± 1 ° C with a temperature-regulated bed (Euthanex Corp.; EZ-212). Post-treatment analgesia (buprenorphine, 0.1 mg/kg; i.m. (I/R) or s.c. (Blunt trauma)) was administered prior to recovery from anesthesia and 12 h post-treatment.

**2.1 Ischemia/Reperfusion Injury**

Ischemia/reperfusion was performed on fourteen (N=14) male Sprague-Dawley rats (Harlan, Indianapolis, IN). First, both hindlimbs were shaved, and one was randomly selected by coin toss. A pneumatic digital tourniquet (D. E. Hokanson, Inc., mod. DC 1.6) was placed (but not inflated) as proximal as possible around the selected leg. Exsanguination of the leg was performed by elevation for 5 min prior to inflation. The cuff was then inflated and maintained at 250 mm Hg using a cuff inflator and air source (D. E. Hokanson, Inc., mod. E20 and AG101), and left in place for 3 h while animals were under anesthesia. Following the ischemic period, anesthesia was withdrawn and the animals were allowed to recover. Animals were subsequently euthanized (sodium pentobarbital, i.p. 150 mg/kg; 21 gauge needle) 2 h post-injury and leg muscles (healthy and injured) were excised, cleaned of fascia and connective tissue then weighed. Immediately following, EDL muscle samples were snap frozen in liquid $N_2$ and stored at -80 ºC.

**2.2 Blunt Trauma Injury**

To study the effects of a freeze-induced (Blunt force/freeze trauma) injury on skeletal muscle markers of interest, sixty (N=60), male, C57BL/6J mice (10 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). Freeze injury was performed in the left, rear hind limb. The non-injured contralateral leg served as one control. Since MT production from the liver may confound the physiology of the non-injured contralateral leg, TA muscles from an additional group of animals were harvested to serve as a second-level of control. These control animals received a sham surgical procedure on one leg and the contralateral leg served as a control.

Prior to freeze-injury, the left hind limb was shaved and the TA muscle was exposed via a 1 cm long incision in the aseptically-prepared skin overlying the TA muscle. The injury was induced by applying a 6 mm diameter steel probe (cooled to the temperature of dry ice) to the belly of the TA muscle (directly below incision site) for 10 seconds. Following injury, the skin incision was closed using 6-0 plain gut absorbable suture, with a 3/8 circle/10.5mm needle (Ethicon, Piscataway, NJ). Following the procedure, anesthesia was withdrawn and mice recovered on a heating pad. Mice were euthanized using $CO_2$ inhalation and cardiac puncture at 3, 10, 24, 48 and 72 h post-injury. Muscles from the hind limbs were excised, cleaned of fascia and connective tissue then weighed. Immediately following, TA muscle samples were snap frozen in liquid $N_2$ and stored at -80 ºC.

**2.3 Tissue Analysis**

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) and immunoblotting were used to quantify MT, MMP-2, MMP-3, and MMP-9 mRNA and protein, respectively. Immunohistochemistry was used to confirm immunoblotting results and to localize protein products in skeletal muscle samples.

For the qRT-PCR, total RNA was isolated from ~10 mg muscle samples and purified according to manufacturer's instructions using TRI Reagent® (St. Louis, MO). RNA quantity and integrity was measured on a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Equal amounts of total RNA were then synthesized into cDNA (Fermentas, Hanover, MD). ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all PCR protocols. Forward and Reverse qRT-PCR Primers (Table 1) for transcripts of interest were designed using NCBI gene sequences and the Primer Design Platform provided by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). NCBI BLAST searches were performed on primer sequences to insure specificity. Melting curve analysis of qRT-PCR products were performed to identify primer dimers. Constitutively expressed genes (Rat: β-Actin; Mouse: TA TA Box binding protein (TBP)) were used as reference standards.

Table 1. Primer Sequences for qRT-PCR

| Gene of Interest/ Species | Forward | Reverse |
|---|---|---|
| MT/ *Rattus Norvegicus* | GCC TTC TTG TCG CTT ACA CC | AGG AGC AGC AGC TCT TCT TG |
| MMP-2/ *Rattus Norvegicus* | ACA CTG GGA CCT GTC ACT CC | AGT GGC TTG GGG TAT CCT CT |
| MMP-9/ *Rattus Norvegicus* | CAC TGT AAC TGG GGG CAA CT | AGG GGA GTC CTC GTG GTA GT |
| B-Actin/ *Rattus Norvegicus* | AGC CAT GTA CGT AGC CAT CC | CTC TCA GCT GTG GTG GTG A |
| MT/ *Mus Musculus* | GCT GTC CTC TAA GCG TCA CC | AAA GAC CAA GGA TCG GGA GT |
| MMP-2/ *Mus Musculus* | CTT CGC TCG TTT CCT TCA AC | AGA GTG AGG AGG GGA ACC AT |
| MMP-3/ *Mus Musculus* | TGG AGA TGC TCA CTT TGA CG | GCC TTG GCT GAG TGG TAG AG |
| TBP/ *Mus Musculus* | ACA TGC CCT TTG AGA TCC GTT TGC | TTT ATC CGA TAG CAC ACG GCA GGA |

Immunoblotting was performed by first homogenizing skeletal muscle samples in buffer [100 mM

Tris, pH 7.4 supplemented with 250 mM sucrose/protease inhibitor mixture (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 µg of leupeptin per ml, 10 µg of aprotinin per ml, 1 µM microcystin-LR, 1 mM phenylmethylsufonyl fluoride, 10 mM sodium fluoride)) followed by centrifugation at 10,000 X g for 30 min at 4 ºC. Supernatants were collected, and analyzed for total protein concentration (Lowry method) using commercially available reagents (Sigma, St. Louis, MO). Equal amounts of supernatant (~30 µg) combined with loading dye were loaded per lane and subjected to SDS-PAGE (sodium dodecyl sulfate-Polyacrylamide gel electrophoresis) using 7 - 15 % gradient gels (Bio-Rad Laboratories, Hercules, CA). Precision Plus Kaleidoscope Protein Standards (Bio-Rad Laboratories) were used as molecular weight markers. Proteins were then electrophoretically transferred to Polyvinylidene Difluoride membranes (GE Healthcare, Piscataway, NJ) and incubated for 1 h at room temperature in a solution of TBS containing 5% nonfat dry milk (Carnation, Solon, OH) or Bovine Serum Albumin (BSA, Sigma) and 0.1 % Tween-20 (TBS-T) (Bio-Rad).

After rinsing, membranes were incubated overnight at 4 ºC in primary antibodies diluted in 3-5 % BSA/TBS-T against MT (1:500, Stressgen, SPA 550E), MMP-2 (1:200, Santa Cruz, sc-13595), MMP-3 (1:100, Santa Cruz, sc-6839), or MMP-9 (1:100, Santa Cruz, sc-6841). Following three rinses in TBS-T, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies (1:1000-1:10,000, 5 % BSA/TBS-T, Bio-Rad) for 1 h at room temperature. After three washes in TBS-T, antibody binding was detected using SuperSignal West Pico Substrate (Thermo Scientific, Rockford, IL) for chemiluminescence. For MT analysis, tissue lysates were heat-treated, and then reduced and alkylated prior to silver-stain analysis (Kimura et al. 1991). 20 ug total protein was loaded per lane on 18% Tris-HCl gels. The intensity of bands from all immunoblotting procedures were quantified using GeneSnap image analysis software (Syngene, Frederick, MD).

Immunohistochemical analysis was performed on a subsample of tissue samples to confirm immunoblotting results and to identify location of proteins of interest. Frozen skeletal muscle samples were cut into three equal sections, positioned for cross-sectional cutting, and immersed in O.C.T. embedding medium (Fisher Scientific, Pittsburgh, PA). Ten to 12 µm slices were generated on a CompPro 3000 Cryostat at -25 ºC (Vibratome, St. Louis, MO) and placed on glass slides coated with Vectabound reagent (Vector Laboratories, Burlingame, CA). Sections were then washed in phosphate-buffered saline (PBS), pH 7.4 (Sigma), blocked with goat normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA) or rabbit normal serum (Zymed Laboratories, San Francisco, CA) and stained for 1 h at room temperature with primary antibodies used for immunoblotting, diluted 1:250-1:500 in 5 % BSA/PBS. Slides were washed in PBS and incubated for 30 min at room temperature with secondary fluorochrome-labeled antibodies (Molecular Probes, Eugene, OR). After washing with PBS, Vectashield® Mounting Medium with 4',6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and a cover slip were added.

All samples were visualized and photographed using a Nikon TI-U inverted microscope equipped with a Retiga 2000R camera and NIS-Elements image analysis software (Nikon Corporation, Tokyo, Japan). Exposure time and brightness were kept constant during analysis. For negative controls, only secondary antibody was used.

### 2.4 Statistical Analysis

Statistical analysis was conducted using the SPSS statistical package version 13.0 (SPSS Inc., Chicago, IL). Either a paired T-test or a repeated measures ANOVA was used to compare mRNA and protein data from injured- vs. healthy or sham muscle over time. Alpha was set at 0.05. A significant ANOVA finding was followed up with appropriate post-hoc tests.

## 3. RESULTS

### 3.1 Ischemia/Reperfusion Injury Results

All data are presented as means ± standard error (SE). I/R resulted in an upregulation of MT and MMP-9 mRNA compared to control ($p<0.05$), with no change in MMP-2 (Figure 1).

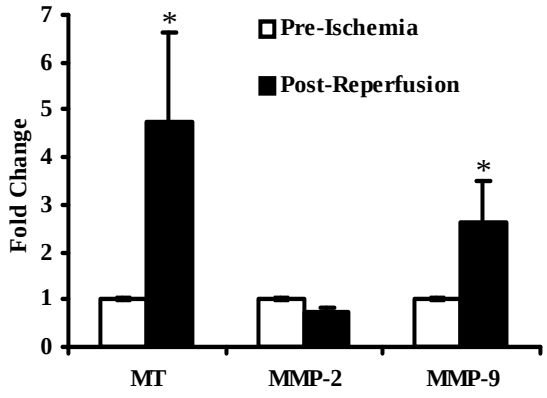


Figure 1. MT and MMP-2 and MMP-9 mRNA expression pre- and post- ischemia/ reperfusion injury. * = $p<0.05$

Immunoblot analysis revealed that protein levels of MT were increased as a result of I/R injury ($p<0.05$) (Figure 2). No changes were observed in protein levels for

MMP-2 or MMP-9 (Data not shown). As an exploratory aim, we also investigated phosphorylation status of the mTOR protein synthesis pathway to understand the effects of this particular injury on acute changes in protein turnover. Immunoblotting data indicated that the mTOR protein level, its phosphorylation on Ser2448, and the activity of S6K1 were unaffected post-I/R injury, indicating that this injury did not affect acute regulation of protein synthesis (Data not shown).

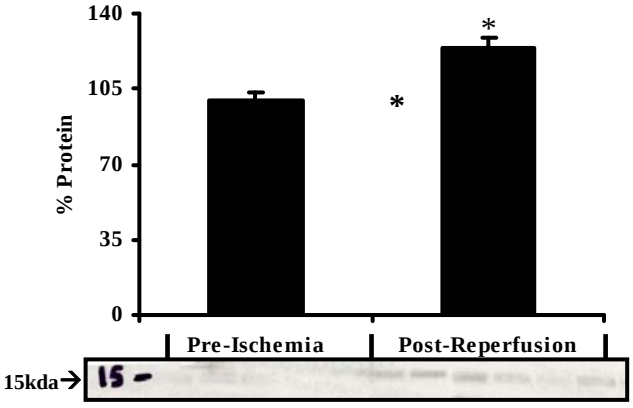


Figure 2. MT protein levels pre- ischemia and post- reperfusion injury. * = $p<0.05$

Immunohistochemistry confirmed results of immunoblotting (Figure 3). Skeletal muscle samples probed for MT protein pre-ischemia showed very little staining for metallothionein. In contrast, MT protein expression increased post-reperfusion.

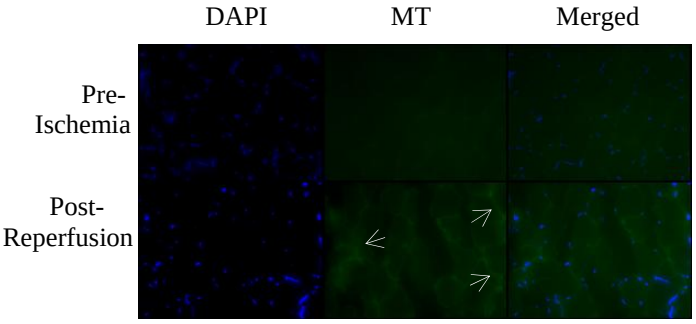


Figure 3. Immunohistochemical analysis of MT. Blue staining (DAPI) represents nuclei and infiltrating neutrophils and macrophages. Green staining represents MT. Increased MT staining was seen in membrane regions of myofibers post-reperfusion (white arrows).

### 3.2 Blunt Trauma Injury Results

Blunt force trauma/freeze injury resulted in an increase in mRNA expression at 3h, 10h, 24h, 48h, and 72h, respectively for MT (2.15-, 3.43-, 4.23-, 3.50-, 1.2 (n.s.)- fold), MMP-2 (1.25-(n.s), 1.28-(n.s), 1.07-(n.s), 1.84- and 1.86-fold), and MMP-3 (4.70-, 67.68-, 40.04-, 24.29, and 14.0-fold) post-injury (Figure 4).

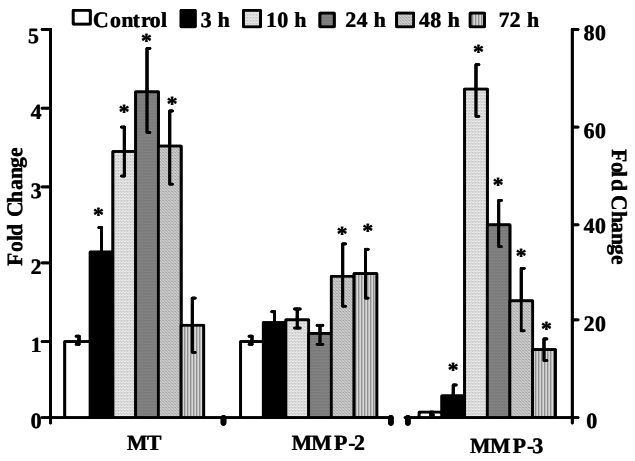


Figure 4. MT, MMP-2 and MMP-3 mRNA expression in uninjured (control) TA muscles versus injured muscle (3 – 72 h post-injury). * = $p<0.05$ versus control leg.

Immunoblot analysis of protein levels of MT and MMP-2 correlated well with mRNA data. The hexameric form of MT protein was increased 14.5-fold 24 h post-injury ($p<0.05$). In regards to MMP-2, the pro- form of the enzyme increased 218 %, 260 %, 321 %, 191 %, and 289 % at 3 h, 10 h, 24 h, 48 h, and 72 h post-injury, respectively, versus the uninjured leg ($p<0.05$). In contrast, the active form of MMP-3 (28/29 kDa) decreased in comparison to the uninjured leg in the days post-injury (Figure 5), while total MMP-3 protein increased approximately 2.5-fold post-injury.

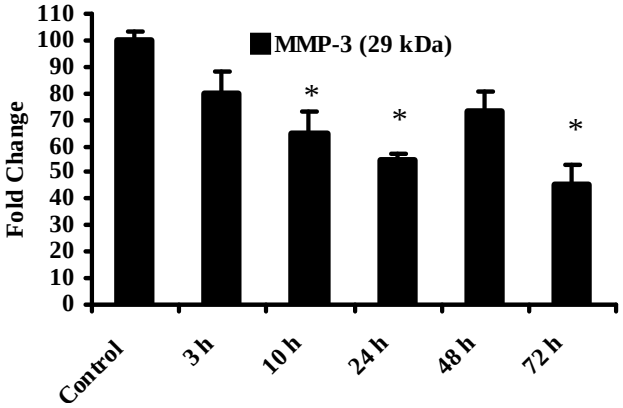


Figure 5. Immunoblot analysis of active (28/29 kDa) MMP-3 in injured TA muscle as compared to uninjured (control) TA 3-72 h post-injury. Injury resulted in a significant down regulation of the active MMP-3 enzyme as compared to control legs at 10, 24 and 72 h post-injury (*$p<0.05$).

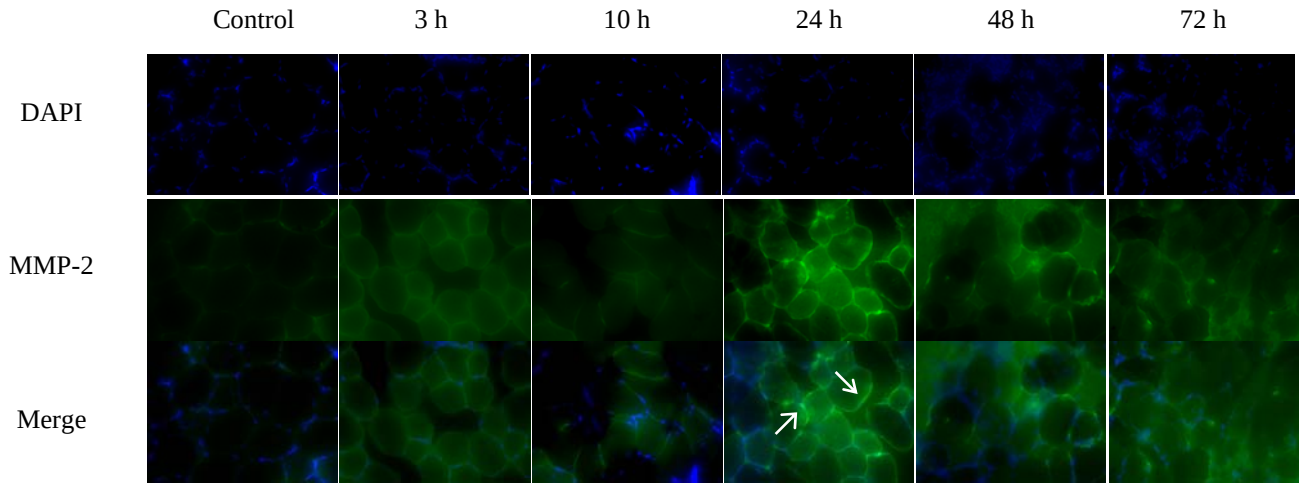


Figure 6. Immunohistochemical analysis of MMP-2 in uninjured (control) and injured TA muscles 3 – 72 h post-injury. Blue staining (DAPI) represents nuclei, and highlights infiltrating neutrophils and macrophages at 48 and 72 h post-injury (top panel). Green staining (middle panel) represents MMP-2 protein. The bottom panel is the merged image of DAPI and MMP-2. Increased MMP-2 staining was seen in the membrane region of myofibers post-injury, with the most pronounced increase in staining 24 h post-injury (white arrows).

Immunohistochemistry was performed on TA muscle samples from uninjured (control) and injured legs from each of the time points post-injury. In agreement with immunoblotting results immunohistochemistry for MMP-2 increased in the days following injury with peak staining at 24 h post-injury (Figure 6). Despite the decrease in the active form of the MMP-3 enzyme, in agreement with immunoblotting, immunohistochemistry for total MMP-3 protein identified a significant increase in staining for MMP-3 in the days post-injury. Results of immunostaining also confirmed immunoblotting results for MT for tissues from freeze injury (Data not shown).

## 4. DISCUSSION

The aim of our work was to investigate the involvement of MT, MMP-2, MMP-3 and MMP-9 in two specific military-relevant injury models: I/R and blunt trauma injury. In agreement with our hypothesis, in both models, we documented an increase in MT mRNA and protein products, and a subsequent increase in MMPs at later time points, specifically during the remodeling phase (24- 72 h post-injury). Also in agreement with our hypothesis were our data from immunohistochemistry analysis identifying an increase in MT and MMP proteins at the extracellular matrix. These findings suggest that MT and MMPs play critical roles in orchestrating extracellular matrix protection and remodeling, respectively, in the hours and days following injury.

Our results clearly show that the type and magnitude of injury regulate the MT and MMP response. We show that the rate of increase in mRNA and protein products is contingent upon the magnitude or severity of the injury. More importantly, we have found that the type of injury impacts the initial MMP enzyme response at the transcriptional level, likely reflecting the substrate most affected by the nature of the injury. For example, the I/R injury had the greatest impact on the MMP-9 Gelatinase whose major substrates include proteoglycans, elastin, fibronectin and collagens (COL) IV, V, and VII. In contrast, with the blunt trauma injury, the most robust change was found in MMP-3 mRNA. Major substrates of MMP-3 include those targets of MMP-9 (proteoglycans, fibronectin and COL IV and COL V) but also gelatin, pro-MMP-1 and COL III and IX (Kjaer 2004). Although both enzymes are induced by the inflammatory mediators TNF-α and IL-1β, and both are able to degrade COL IV to facilitate the degradation of basement membranes, our results indicate that the pathology of the injury regulates the specific form of MMP released. Thus, it is plausible that the specificity of the injury-response, at least in regards to the MMPs, is regulated by the injury itself.

Factors contributing to the observed increase in MT are not well characterized in the literature, particularly in skeletal muscle tissue. Our immunohistochemistry data show (Figure 3) MT expression in healthy skeletal muscle is sparse. In response to injury, however, as demonstrated here in I/R and blunt trauma injury, and by others in response to exercise stress or spinal cord injury (Penkowa et al. 2005; Urso et al. 2007) MT expression increases at the transcriptional level within the first few hours following injury or stress, with a subsequent increase in protein products in the days following injury. Several lines of evidence signify that metallothioneins are induced in stressed or injured tissue in response to ROS induction, primarily following membrane damage (Bobillier Chaumont et al. 2001; Feng et al. 2006; Kondo et al. 1992; Scheede-Bergdahl et al. 2005; Tapiero; Tew 2003).

Based on the results of the experiments outlined here, and the aforementioned studies, it is tempting to

speculate that membrane damage incurred as a result of both injuries induces the ROS-mediated cascades. In turn, MT production is increased to scavenge free radicals released in response to injury and reperfusion (Penkowa et al. 2005; Scheede-Bergdahl et al. 2005). If this is the case, MT is likely to play a protective role in skeletal muscle in the hours and days following injury. Indeed, this relationship asserts the benefits of pharmacologically-induced MT production to confer protection at the level of the extracellular matrix in the first few hours following injury. We have designed future experiments to explore the efficacy of MT as a countermeasure to offer protection to the extracellular matrix, and possibly minimize injury. This is a critical area of exploration, however, certain factors must be considered. Primarily, it is important to characterize the effects of high levels of MT administered exogenously on the in vivo production of other molecules critical to skeletal muscle remodeling. Additionally, it is not yet known if increased MT expression affects transcription of downstream regulators of protein synthesis and extracellular matrix remodeling.

To this end, the effects of injury on transcriptional and pre- and post- translational responses of the MMP system are important to consider as they offer an additional line of intervention when exploring pharmacological countermeasures (Brown et al. 2004). The effects of MMP induction (MMP-2 and MMP-9) at the protein level, and the decrease in the active form of MMP-3 as was seen in the blunt trauma model, indicate that severe injury affects extracellular matrix integrity, as well as the ability to efficiently maintain extracellular matrix turnover. Decreased presence of the active form of MMP-3 may be problematic in the regeneration phase, impeding proper muscle healing resulting in altered muscle function post-recovery (Carrell et al. 2002).

Moreover, the excessive production of MMP-3 and MMP-9, at least at the transcriptional level, provides evidence that the damaged membrane is further compromised. An important regulatory step in the activation of other MMPs involves the expression and activational state of MMP-3 (Spinale et al. 2000). Therefore, it is likely that the increase in total MMP-3 protein in the days following injury activates other latent proteolytic enzymes. The effect of this response might include further-compromised membrane integrity, decreased protein synthesis, increased protein breakdown, and an overall delay in the healing process. Additionally, upon recovery, the structural integrity of the membrane may render the muscle unable to sustain certain stressors, increasing the risk of subsequent injury.

Thus, pharmacological inhibition or the attenuation of specific MMP induction and downstream proteolytic cascades has the potential to prevent additional injury, while reducing the time to recovery post-trauma. This work is seminal in deciphering the complex alterations in signaling pathways following a traumatic injury to skeletal muscle. With the data we present here which characterizes those molecules most affected in the first few hours post-injury, we are better suited to design pharmacological interventions to modulate these responses.

For instance, we are currently investigating the effects of an Adenosine Receptor type-3 (A3) agonist as an effective countermeasure in response to injury. A recent investigation by Zheng and colleagues (Zheng et al. 2007) has shown that pre-treatment with the A3 agonist significantly attenuates the degree of reperfusion-induced skeletal muscle injury following 2h of ischemia. A3 receptor agonists are now being tested with various other military-relevant skeletal muscle injury models to assess their efficacy in minimizing skeletal muscle injury and maintaining skeletal muscle function.

## 5. CONCLUSIONS

MT production is likely related to injury-induced oxidative-stress, which potentially affects extracellular matrix integrity, explaining our observed increase in the MMPs. These data suggest that oxidative-mediated extracellular matrix disruption is a potential mechanism for increased skeletal muscle proteolysis in response to injury. A suitable countermeasure to either increase MT induction or to attenuate the upregulation of the MMPs would be one that affords protection to the extracellular matrix.

From the results of this work, we have designed future studies to explore the potential effects of a pharmacological intervention in preventing reactive compounds from interfering with compounds involved in the repair process. This work is relevant to the military because results will help us to understand the molecular response to injury and identify means to minimize damage and facilitate a more rapid recovery in skeletal muscle. Additionally, minimizing the detrimental effects of proteolytic cascades on membrane repair and integrity will insure that once skeletal muscle is healed, the tissue integrity has not been compromised and individuals are not at increased risk for injury.

## DISCLAIMER

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals" as

prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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