

The Combined Influence of Hemorrhage and Tourniquet Application on the Recovery of Muscle Function in Rats

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Objective: The objective of this study was to compare the effect of tourniquet-induced ischemia/reperfusion (I/R) injury on the recovery of muscle function with and without prior hemorrhage.

Methods: Male Sprague-Dawley rats (initially 400–450 g) were randomly assigned to 1 of 4 groups (n = 8 per group): (1) hemorrhage (33% of estimated blood volume) plus tourniquet (+H/+TK); (2) tourniquet alone (−H/+TK); (3) hemorrhage alone (+H/−TK); and (4) surgical control (−H/−TK). A pneumatic tourniquet was applied to the upper leg for 4 hours, followed by 2 weeks of recovery. For +H animals, tourniquets were applied at the conclusion of blood withdrawal. The predominantly fast-twitch plantaris and the predominantly slow-twitch soleus muscles were examined using in situ isometric muscle function 2 weeks following treatment.

Results: Tourniquet application resulted in significantly greater loss of force production [peak tetanic force (Po)] in the plantaris compared with the soleus. The decrease in Po was a result of both a loss of muscle mass and a reduction in specific force [force per unit weight; Po (n/g)]. Hemorrhage prior to tourniquet application significantly increased the extent of functional loss compared with tourniquet alone in the plantaris but not the soleus. Hemorrhage prior to tourniquet application significantly reduced the rate of postsurgical recovery of body weight.

Conclusion: The functional loss resulting from tourniquet application is exacerbated by the superimposition of hemorrhage in the predominantly fast-twitch plantaris but not the predominantly slow-twitch soleus. This was likely a result of metabolic derangement resulting from the combination of hemorrhage and tourniquet application. The development of interventions designed to attenuate the loss of muscle mass and function following complex trauma is necessary for optimal patient recovery.

Key Words: fiber type, healing, ischemia reperfusion injury, rat, regeneration, repair, skeletal muscle

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INTRODUCTION

Wounds to the extremities are the most frequently encountered in combat, accounting for between 60% and 80% of injuries on the modern battlefield.^{1–5} These injuries are frequently severe and often accompanied by major vascular damage, making them a leading contributor to preventable battlefield mortality.^{6,7} Tourniquets are the most effective way of treating severe arterial trauma in extremities under emergency combat conditions and have saved countless lives in the current wars in Iraq and Afghanistan (personal communications Col. John Holcomb, MC, and Col. John F. Kragh, MC). Furthermore the liberalization of tourniquet use^{8–10} and the availability of effective, easy-to-use, combat tourniquets have greatly increased tourniquet use.^{8,9} Regardless of how rapidly a tourniquet is applied, considerable blood loss can occur before control of hemorrhage is achieved.

Tourniquet-induced ischemia/reperfusion (I/R) injury results in a complex cascade of responses that can lead to muscle degeneration and loss of muscle function.^{11–13} Severe hemorrhage results in global I/R, which can lead to multiorgan system failure; even in less severe cases these organ systems undergo some degree of I/R.¹⁴ It is unknown whether the combinations of these 2 physiologic stressors interact to worsen muscle injury.

Previous studies from our laboratory have shown that hemorrhage (33% estimated blood volume) combined with tourniquet application paradoxically reduces muscle edema and reduces loss of viability acutely (2 hour and 48 hour) as assessed by histology and vital staining.¹⁵ However, it is unknown whether this acute beneficial effect translates to greater return of muscle function during recovery at later time points. Here we report the combined impact of hemorrhage and tourniquet application on the rate of recovery of muscle function.

METHODS

Male Sprague-Dawley rats were obtained from the colonies of the Charles River (Wilmington, Massachusetts). At the time of the experiment rats weighed between 400 and 450 g. Rats were randomly assigned to 1 of 4 treatment groups (n = 8 rats/group): (1) hemorrhage (H) and tourniquet

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application (+H/+TK); (2) tourniquet alone (−H/+TK); (3) hemorrhage alone (+H/−TK); or (4) no hemorrhage and no tourniquet (−H/−TK; surgical control). All treatment groups underwent the same experimental analysis. All animal protocols were approved by the United States Army Institute of Surgical Research Animal Care and Use Committee. Animals were housed in a vivarium accredited by the American Association for the Accreditation of Laboratory Animal Care. Animals were provided with food and water ad libitum prior to and following all procedures.

All procedures were performed under 1.5% to 2.5% isoflurane anesthesia, adjusted to maintain a surgical plane. All rats received carotid catheterization immediately prior to the treatment. The right carotid artery was isolated and a catheter was inserted and held in place with 4-0 ligatures for blood withdrawal. Hemorrhage took place over a 5-minute period and corresponded to 33% of total blood volume (estimation based on 64 mL blood/Kg body weight). To approximate hemorrhage on the battlefield initial pilot studies were performed to determine the maximal nonlethal hemorrhage volume and bleeding rate that could be consistently achieved across animals.

During the hemorrhage and 5 minutes prior to tourniquet application the leg was exsanguinated by elevating it above the level of the heart. Exsanguination was performed to minimize pooling of blood following tourniquet application that may lead to thrombotic complications and to mimic the exsanguination that takes place following the vascular trauma indicating tourniquet application. A pneumatic digit tourniquet attached to a tourniquet regulation system was placed as proximal as possible around the upper limb and inflated to a pressure of 250 mm Hg for 4 hours. The use of 250 mm Hg was based on pilot studies using orthogonal polarizing spectral imaging (Cytometrics, Bridleways, England) to image blood flow. These studies found that 220 mm Hg effectively eliminated microvascular flow. We then chose to add a safety factor of 30 mm Hg to this. Four hours of tourniquet application was chosen based on estimates of the time of wounding to the time of definitive medical care on a dynamic battlefield. During this time the catheter was removed, the artery was tied off, and the muscle and skin were closed in layers with 4-0 ligatures. All procedures were done under aseptic conditions. Following tourniquet application the rats were returned to their cages. Analgesia (buprenorphine, 0.1 mg/kg IP) was administered prior to withdrawal of anesthesia and at 12-hour intervals for the first 24 hours.

Two weeks following tourniquet application muscle function was measured in situ according to previously published methods and procedures.¹⁶ Isometric measurements were made on the predominantly fast-twitch plantaris muscle and predominantly slow-twitch soleus muscle. Activation of the muscle was accomplished via stimulation of the motor nerve. Contractile properties were measured using a dual-mode servo muscle lever system (Aurora Scientific, Mod. 305b and 305b-LR for the soleus and plantaris, respectively). A PC loaded with a Labview (National Instruments, Inc, Austin, Texas)-based program controlled the muscle lever and stimulator, recorded all signals (2000 Hz), and performed real-time analysis of the force and length signals. The nerve was

stimulated at 2 times the voltage required to elicit maximal peak twitch tension (Pt) at a pulse width of 50 μ s. All measurements were made with the muscles set at Lo, defined as the Pt established from a series of twitches at increasing muscle lengths (0.2-mm increments). Following establishment of Lo, Pt was determined from the average of 3 unpotentiated twitches (2 minutes between each twitch); peak tetanic force (Po) production was determined from the average of 3 tetani separated by 2 minutes. Stimulus frequency for Po was set at 100 Hz for the soleus and 150 Hz for the plantaris.

Following determination of contractile properties, rats were euthanized with a 1.0-mL injection (IP) of Fatal Plus. The muscles were excised, trimmed of connective tissue, blotted dry with filter paper, and weighed. They were then stretched to Lo, pinned to a tongue depressor, and fixed in 10% buffered formalin solution (Fisher Scientific, Pittsburgh, Pennsylvania). After fixation, muscles were embedded in paraffin, cross-sections (8- μ m thick) were cut from the muscle belly (widest portion) using a microtome, and they were stained with hematoxylin and eosin (H&E). The stained sections were graded by a veterinary pathologist who was blind to muscle and treatment. Grading was done using a 5-point scale, with 0 representing no apparent pathology and 4 signifying extreme pathology. Grading categories included myofiber degeneration and necrosis, small fibers, large regenerating fibers, fibrosis, inflammation, edema, and vasculitis.

Comparisons were made between (1) +H/+TK versus −H/+TK (*effect of hemorrhage + tourniquet*); and (2) +H/−TK versus −H/−TK (*effect of hemorrhage alone*). Continuous data were analyzed using a Student's *t*-test. Pathology grading was analyzed using the Mann-Whitney rank sum test for comparisons of group medians. Differences were considered to be significant at $P < 0.05$. All data are presented as mean $\pm$ standard error of the mean.

RESULTS

Body Weight

All groups underwent a reduction in body weight immediately after treatment (Fig. 1). +H/−TK and −H/−TK recovered in a similar pattern, reaching within 10g of pretreatment values by day 10 and attaining pretreatment values by day 14. −H/+TK reached near pretreatment values but at a slower rate. +H/+TK declined to similar value as −H/+TK immediately after treatment; however, the rate of weight gain was significantly ($P < 0.05$) less for +H/+TK and did not reach pretreatment values by day 14.

Muscle Weight

Tourniquet application resulted in a loss of mass in the plantaris and soleus in both +H/+TK and −H/+TK groups (Fig. 2). When compared with −H/−TK, +H/+TK was 34% and 19% lighter for the plantaris and soleus, respectively. When compared with −H/−TK, −H/+TK was 19% and 6% less for the plantaris and soleus, respectively. Both the plantaris and soleus were approximately 18% less for +H/+TK compared with −H/+TK; however, this was not statistically significant ($P > 0.05$). Hemorrhage alone (+H/−TK versus −H/+TK) had no significant ($P > 0.05$) impact on muscle mass.

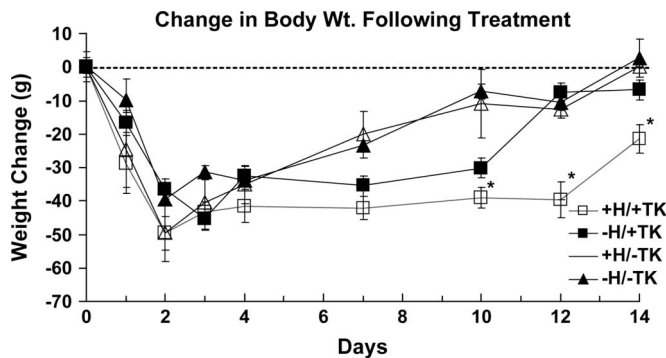


FIGURE 1. Body weight curves spanning the duration of the study. The initial body weights were 439, 421, 419, and 432 g for +H/+TK, -H/+TK, +H/-TK, and -H/-TK, respectively. *Significantly different from -H/+TK ($P < 0.05$).

Contractile Properties

Four hours of tourniquet application resulted in a large decrease in Po (Fig. 3). The impact was greater in the plantaris (5-fold decline) (Fig. 3a) than in the soleus (3-fold decline; Fig. 3b). Hemorrhage prior to tourniquet application significantly ($P < 0.05$) compounded the decline in Po in the plantaris but had no impact on the soleus. Hemorrhage alone had no impact on force generation (-H/-TK versus +H/-TK). Specific Po (n/g muscle weight) declined in both plantaris (Fig. 3c) and soleus (Fig. 3d) as a result of tourniquet application. Compared with -H/-TK, the decline in specific Po was 4- and 8-fold in the plantaris for -H/+TK and +H/+TK, respectively, and 2-fold in the soleus for both tourniquet groups. The decline in Po (n/g) was significantly ($P < 0.05$) greater when hemorrhage was combined with tourniquet application (+H/+TK versus -H/+TK). The Pt/Po ratio increased similarly among muscles in response to tourniquet application independent of hemorrhage (ie, +H/+TK = -H/+TK; Fig. 3e-f). The increase was a function of the loss of Po because Pt was not significantly ($P > 0.05$) reduced.

Histology

The impact of tourniquet application on histology is clear from examination of Table 1. In general the scores for the plantaris were higher (more pathology) than the soleus. There was no significant difference between the same muscles for +H/+TK and -H/+TK. No apparent pathology was found in either of the nontourniquet groups (+H/-TK, -H/-TK).

DISCUSSION

The main finding of the present study is that the functional loss resulting from tourniquet application is exacerbated by the superimposition of hemorrhage in the predominantly fast-twitch plantaris but not the predominantly slow-twitch soleus. This extends previous work by us and others that have demonstrated that soleus is less sensitive to tourniquet-induced I/R compared with predominantly fast-twitch muscles.²³

The differences between +H/+TK and -H/+TK were not a result of greater initial injury in the +H/+TK. In a previous study using the identical treatment protocol we demonstrated

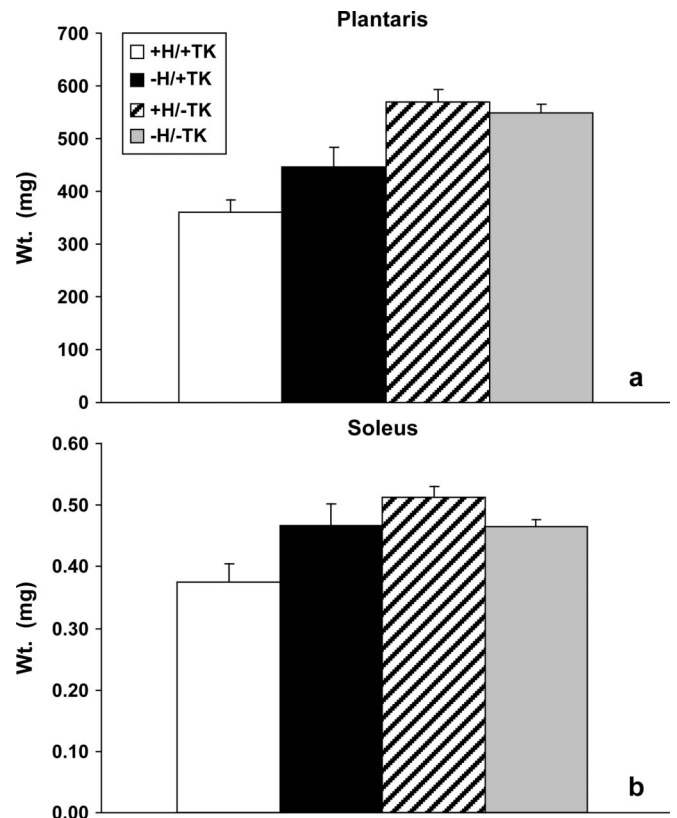


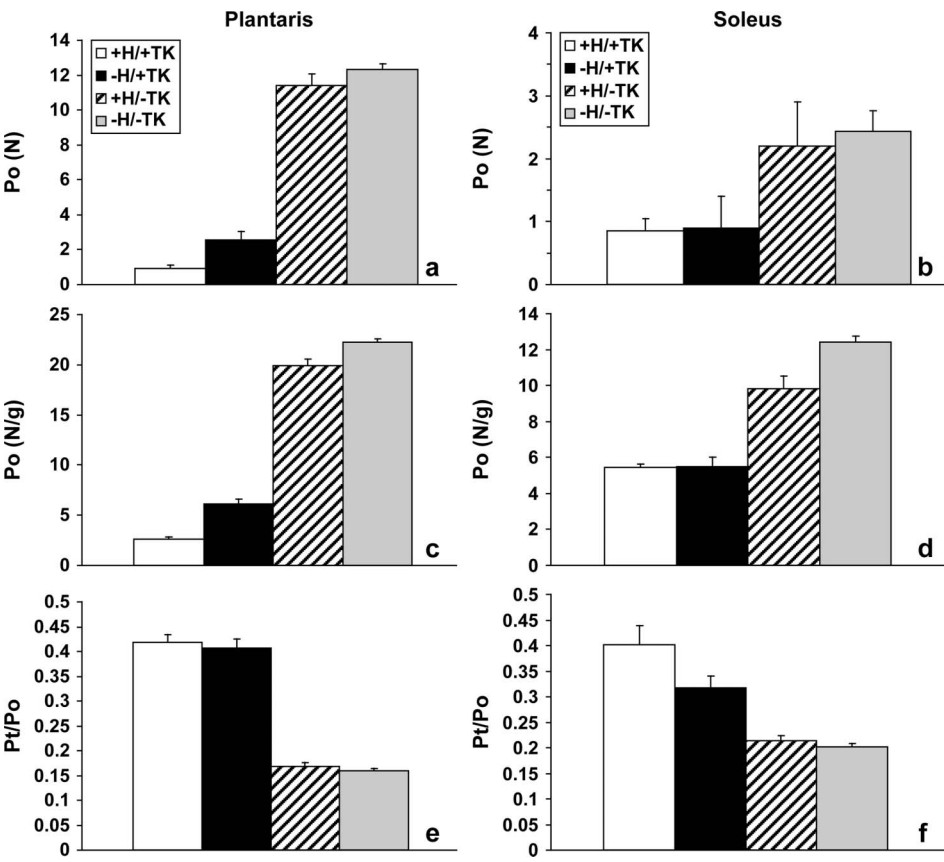
FIGURE 2. Muscle weights at day 14. Tourniquet application resulted in a large decrease in muscle mass in both the plantaris (a) and soleus (b). The difference between +H/+TK and -H/+TK was not statistically different for either muscle.

that 2 hours or 2-days postinjury there was no difference in muscle viability based in the vital stain nitroblue tetrazolium (NBT), and edema was significantly less in the +H/+TK groups.¹⁵ Thus the difference between the tourniquet groups in the present study must have been a result of a slower rate of healing when hemorrhage was added.

Examination of the body weight curves (Fig. 1) shows that weight loss following tourniquet application was similar for both +H and -H groups, reaching a nadir at day 2 with identical weight loss among groups at that time point. However, the rate of recovery is significantly reduced with the addition of hemorrhage (+H/+TK) and remained significantly less than all other groups at day 14. It cannot be determined from the current data why this occurred, but it is likely a result of reduced caloric intake and/or trauma-induced metabolic derangement. We have recently shown similar differences in the pattern of weight loss and recovery between burn-injured and sham rats.¹⁷ With burn-injured rats the pattern was shown to be the result of burn-induced hypermetabolism, as caloric intake was matched between burn and sham rats. Regardless, the blunted recovery of weight is an index of the health status of the animals. It is well-known that health status has a potent impact on healing in general.

The reduced Po in the +TK groups (Fig. 3a and b) was a result of both a loss of muscle mass (Fig. 2) and a loss of

FIGURE 3. Isometric force measurements for the plantaris (a–c) and the soleus (d–f) determined 14 days posttreatment. Statistical comparisons were made between +H/+TK versus –H/+TK (effect of hemorrhage + tourniquet) and +H/–TK versus –H/–TK (effect of hemorrhage alone). Specific Po (c and d) is expressed relative to muscle wet weight (g). The combination of hemorrhage and tourniquet resulted in a significant decrease in Po (N) and Po (N/g) in the predominantly fast-twitch plantaris (a, c; $P < 0.05$) but had no significant impact on the predominantly slow-twitch soleus (b, d; $P > 0.05$). Hemorrhage alone (+H/–TK versus –H/–TK) had no impact on Po (N or N/g) in either muscle ($P > 0.05$). Tourniquet application resulted in an increase in the Pt/Po ratio (e, f) in both muscles primarily as a result of the decrease in Po.



specific force (Po n/g) (Fig. 3c and d) and was significantly more pronounced when hemorrhage was combined with tourniquet application. As discussed earlier, this was likely because of the added impact of the hemorrhage on the health status of these animals.

In contrast to the predominantly fast-twitch plantaris, hemorrhage did not result in a greater loss of specific force in the predominantly slow-twitch soleus (Fig. 3d). This, combined with relatively reduced loss of mass and absolute force, demonstrates that predominantly slow-twitch muscle is preferentially spared. Preferential sparing of slow-twitch muscle has also been found in the case of burn injury.^{18–20} Muscle activation is known to reduce protein loss during

starvation.²¹ Slow-twitch muscle is routinely activated during normal cage activity; in contrast, fast-twitch muscle is only activated during ballistic movements or when force requirements are high.²² This suggests the need to further study the impact of muscle activation early during the repair and regeneration processes involved in healing following muscle trauma.

Neuropaxia was observed for the first week following tourniquet application (ie, observation of animal behavior revealed normal use of limb, and foot drop was not apparent). Previous investigations in our lab have demonstrated that force production cannot be elicited via nerve stimulation at day 2 posttourniquet application, but it can be elicited using direct

TABLE 1. Median Pathology Scores for Selected Parameters. All Values are Expressed as the Median and (Range)

Group	Degeneration/Necrosis		Fibrosis		Inflammation		Edema	
	Plant	Sol	Plant	Sol	Plant	Sol	Plant	Sol
+H/+TK	1 (0–3)	1 (0–1)	2 (0–2)	0 (0)	2 (0–2)	0.5 (0–2)	2 (0–2)	1 (0–1)
–H/+TK	1 (1–2)	1 (0–2)	1 (1–2)	0 (0–1)	1 (0–2)	0 (0–2)	1 (0–2)	0 (0–2)
+H/–TK	0 (0)	0 (0)	0 (0)	0 (0)	0.5 (0–1)	0.5 (0–1)	0 (0)	0.5 (0–1)
–H/–TK	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5 (0.5)	0 (0)	0 (0)

muscle stimulation.²³ Nerve injury following tourniquet application has been documented in similar animal models and appears to be caused by compression injury to the motor nerve underlying the tourniquet.^{24–26} In the present study neural stimulation was possible by 14 days; however, it is unknown to what extent this functional denervation interacted with I/R to produce the results observed at day 14.

This study does not entirely mimic the clinical situation in which tourniquets are used. The muscle insult in this experiment does not include a model of the severe limb trauma seen clinically, with disruption of both soft tissue and bone as well as the additional insult of I/R with tourniquet placement. This model is intended to serve as a simple, easily reproducible model of tourniquet-induced skeletal muscle I/R. Because of this limitation, one should be cautious in applying the results of this and other studies using this model to clinical practice. Experiments investigating the combined effect of tissue disruption and I/R are needed and are planned at our institution. Additionally, no resuscitation was provided in this study because it would most often be in clinical care of patients who are severely injured and hemorrhaging. The effects of resuscitation fluids and strategies on skeletal muscle I/R in this model have been examined.²⁷

The major strength of this study was that the assessment of muscle healing was performed with measurements of muscle function; the majority of studies involving muscle injury measure biochemical or histochemical correlates and extrapolate to muscle function. The major limitation was that only one time point is examined; although this provides insight regarding the rate of healing, it does not allow us to determine if both groups ultimately heal to the same extent.

In conclusion, the main finding of the present study is that the functional loss resulting from tourniquet application is exacerbated by the superimposition of hemorrhage in the predominantly fast-twitch plantaris but not the predominantly slow-twitch soleus. This was likely because of metabolic derangement resulting from the combination of hemorrhage and tourniquet application. Understanding the impact of overall health status on muscle healing following injury is critical for optimizing treatments. Additionally, how and why fiber-type composition influences the vulnerability of skeletal muscle to various stressors may provide insights to treating muscle trauma.

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