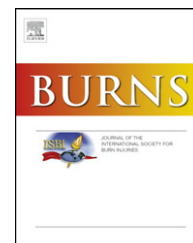


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Muscle contractile properties in severely burned rats^{☆,☆☆}

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ABSTRACT

Burn induces a sustained catabolic response which causes massive loss of muscle mass after injury. A better understanding of the dynamics of muscle wasting and its impact on muscle function is necessary for the development of effective treatments. Male Sprague–Dawley rats underwent either a 40% total body surface area (TBSA) scald burn or sham burn, and were further assigned to subgroups at four time points after injury (days 3, 7, 14 and 21). *In situ* isometric contractile properties were measured including twitch tension (Pt), tetanic tension (Po) and fatigue properties. Body weight decreased in burn and sham groups through day 3, however, body weight in the sham groups recovered and increased over time compared to burned groups, which progressively decreased until day 21 after injury. Significant differences in muscle wet weight and protein weight were found between sham and burn. Significant differences in muscle contractile properties were found at day 14 with lower absolute Po as well as specific Po in burned rats compared to sham. After burn, the muscle twitch tension was significantly higher than the sham at day 21. No significant difference in fatigue properties was found between the groups. This study demonstrates dynamics of muscle atrophy and muscle contractile properties after severe burn; this understanding will aid in the development of approaches designed to reduce the rate and extent of burn induced muscle loss and function.

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1. Introduction

Severe trauma and burn induce a profound hypermetabolic response characterized by increased protein catabolism causing severe loss of lean body mass [1,2], immunologic compromise [3] and slowed wound healing and growth delays [4], all of which contribute to increased morbidity and mortality and prolonged periods of recovery. Catabolism with sustained loss of muscle mass leads to long-term loss of muscle strength and delayed return to customary pre-injury

activities. Although administration of nutrient support during hospitalization has been shown to reduce weight loss in severely burned patients [5] and to decrease loss of body mass in other critically ill patients [6], these reductions are only partial so that severely injured patients still undergo massive wasting of the peripheral musculature with loss of lean body mass [7].

Restoration of normal function is an important outcome after severe burn [8,9]. The time required for burned patients to resume normal function is excessive, dramatically interfering

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with return to normal activities. Even patients with burns <20% of total body surface area (TBSA) require an average of 13 weeks convalescence before they are capable of returning to normal activities and employment [10]. As the percent of TBSA burned increases, so does the amount of time required before returning to normal activities [11]. Retrospective studies indicate that although many burned patients eventually return to normal function, up to one fourth cannot return to previous levels of productivity [9,12], primarily due to the loss of physical capacity for standing or walking. For this reason, successful rehabilitation strategies must optimize muscle strength and function.

While all severely burned patients currently receive directed rehabilitation programs to improve functional outcome during convalescence [13], these efforts are usually begun weeks to months after the wound is healed completely, which is likely to be hampered by loss of muscle mass and therefore strength due to sustained catabolism. We do not currently know whether early treatments devised to improve muscle weight and protein mass benefit muscle function. It is also not clear whether decreased muscle function is associated with proportional loss of muscle mass, or whether injury has independent effects on the contractile properties of muscle. We developed the hypothesis that severe burn in rats is associated with loss of muscle contractile function that is greater than the proportion attributable to loss of muscle mass alone. The ability to understand the dynamics of burn induced muscle loss on muscle function, as well as the ability to develop strategies to reduce early muscle wasting following burn would be aided by a clinically relevant animal model. The goal of this study was to characterize the changes in muscle mass and function following 40% TBSA of burn in a rat model.

2. Methods

Male Sprague-Dawley rats weighing approximately 300 g were used. The study protocol was reviewed and approved by Institutional Animal Care and Use Committees (IACUC) at the United States Army Institute of Surgical Research (USAISR), and the animal study was accomplished in the animal lab facility at the USAISR. The rats were housed in a temperature-controlled environment with a 12-h light/dark cycle. After acclimatization for 1 week, the animals were pair-fed a liquid diet (Boost, Mead Johnson Nutritionals, Evansville, Indiana) and water *ad libitum* for 3 days prior to the experiment, and at daily intervals before the end of experiment. Sixty-four animals were randomly and evenly assigned into burn or sham burn groups. The animals were further assigned to subgroups of four time points at days 3, 7, 14 and 21 after burn ($n = 8$ per subgroup).

Rats were anesthetized by continuous 1.5–3% isoflurane (Forane[®], Baxter Healthcare Corp., IL 60015) in 100% oxygen using a face cone/mask. The rats in both sham and burn groups were shaved on the dorsal and ventral surface of the trunk. The rats in burn group received a 40% total burn surface area (TBSA) by immersing the dorsum in 100 °C heated water for 10 s and ventral surface for 2 s according to the previously established models [15,16]. Burned rats were resuscitated immediately with 20 ml Ringer's lactate solution given intraperitoneally. After burn, the rats were returned to

individual wire-bottom cages, and received analgesia (0.1 mg/kg buprenorphine, Buprenex[®], Hospira, Inc., Lake Forest, IL) subcutaneously twice a day for 3 days. The animals in the sham group received the same analgesic injections. All animals were returned to cages after awakening from anesthesia and pair-fed BOOST[®] at 50 cc of (0.17 kcal/kg body weight) on the first day after burn, 75 cc (0.25 kcal/kg body weight) on the second day, and 100 cc (0.33 kcal/kg) on the third day. Graded daily intake was in accord with the previously defined intake of burned rats [17] which has been verified in several subsequent studies [18,19].

Muscle contractile properties were observed at days 3, 7, 14 and 21 after burn. One leg was randomly chosen from each animal for the test. Following anesthesia by continuous inhalation of isoflurane (1.5–3%), the common sciatic nerve was isolated and implanted into an electrode cuff with ends connected to a pulse stimulator (A-M Systems, Inc., Mod. 2100). After securing the electrode cuff, the proximal end of the nerve was carefully severed. The distal tendon of the tibialis anterior (TA) was isolated and cut at the annular retinaculum dorsal to the hock, and the distal 1/3 TA was gently dissected free from the surrounding musculature leaving the origin and neurovascular pedicle intact. The distal tendon was threaded through a hole in the lever arm of a dual-mode servo muscle lever system (Aurora Scientific, Inc., Mod. 309b) and secured with 4-0 silk suture. The lower leg was secured and stabilized on the working platform with pins at the knee and ankle joints. Core temperature was monitored using a rectal thermistor and maintained at 36.5–37.5 °C by manually adjusting the temperature of circulating water in the surgical platform. The temperature of the peroneus longus muscle was monitored with a needle thermistor, and acted as a surrogate for the TA with maintenance at 36.0 ± 1.0 °C. The isometric contractile properties of the TA muscle were then evaluated using an *in situ* preparation and test battery described previously [20,21]. All measurements were made with the muscles set at optimal length (L_0), which was determined from P_t using an automated routine as follows: at a slack position the muscle was stimulated at 1 Hz for a set of eight twitches; the last two twitches were averaged and the P_t was stored. The lever was then moved 0.1 mm, and the routine was repeated 2 s later. Each twitch set including lever movement took 10 s. This continued until the average P_t did not change more than 2% between three consecutive twitch sets. Optimal length was defined as the second of the three twitch sets. Stimulus frequency for P_0 was set at 150 Hz. Following establishment of L_0 , P_t was determined from the average of three unpotentiated twitches (1 min between each twitch); P_0 was determined from the average of three tetani separated by 2 min. Lastly, the isometric fatigue properties were determined [22] by stimulation at 40 Hz for 330 ms every second for 4 min. Muscle fatigability was defined by the fatigue index calculated by the percent rate of the last tetanic force against initial peak force.

The TA from contra-lateral leg was isolated immediately before muscle function testing for measurement of total muscle wet weight. For wet weight/dry weight determination, a small piece of muscle sample was dissected, weighed and placed in a drying oven at 50 °C for 5 days and weighed again for its dry weight. Another sample of TA (about 100 g) was

processed for whole protein extraction. Briefly, the muscle was snap-frozen in liquid nitrogen and pulverized in BioPulverizer™. The sample was then immediately homogenized in lysis buffer (Cell Signaling Technology®, Danvers, MA) containing 20 mM Tris-HCL, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, and 1 μ g/ml leupeptin. The protein suspension was extracted after centrifugation, and protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL). The total amount of protein and percentage of protein content (protein weight vs. muscle weight) in TA were then calculated.

All data are expressed by mean $\pm$ standard error of mean (SEM). Two-way ANOVA was used to analyze differences between groups. Student's t-test was used to analyze differences between groups at single time points only where mentioned. Significance was established at $p < 0.05$.

3. Results

Both burn and sham animals lost approximately 5% of their original weight by day 3. Sham animals began to regain weight after day 7. Weight loss continued in burned animals by 10–15% until day 14 after burn after which they began to regain weight. At day 21, weights were still approximately 5% less than original weights (Fig. 1A). Sham animals lost less weight than burn rats at day 7, and the body weight increased thereafter following the normal growth pattern, increasing by 10–15% over original weight at day 21. Therefore, both body weight and percentage of body weight change differed significantly between sham and burn groups at 7, 14 and 21 days after burn (Fig. 1A).

TA wet weights in burned rats decreased after burn (Fig. 1B). Significant differences in wet weights between burn and sham groups were found from day 3 to day 21. Compared to the sham group, percent reduction in TA muscle wet weight after burn was –9.7%, –7.4%, –16.0%, and –9.9% at days 3, 7, 14, and 21 respectively. In addition, no significant difference was found in TA dry/wet weight ratios at any time point (Fig. 1C) suggesting no significant difference in tissue water content between the groups. However, a significant difference in dry weights between burn and sham was found at day 7 through day 21.

Total TA protein amount was significantly lower in burn compared to sham at days 7, 14 and 21 after injury (Fig. 2). However, we found no significant difference in TA protein content percent between the groups. At day 14, an 11% reduction in protein content percent was found in burn compared to sham, but this was not statistically significant ($p < 0.067$ by two-way ANOVA) (Fig. 2).

Absolute Pt (twitch tension), Po (tetanic tension), and fatigue index were measured, and the specific force of Pt and Po were expressed by the ratio of Pt or Po to the muscle wet weight of TA (N/g). Pt was not significantly different between the groups from day 3 through day 14, but a significantly higher Pt was found at day 21 in the burn group compared to the sham in both absolute and specific force (Table 1). In addition, we found a slower time to peak tension (greater TPT) and longer time to relaxation in the burn group (greater RT)

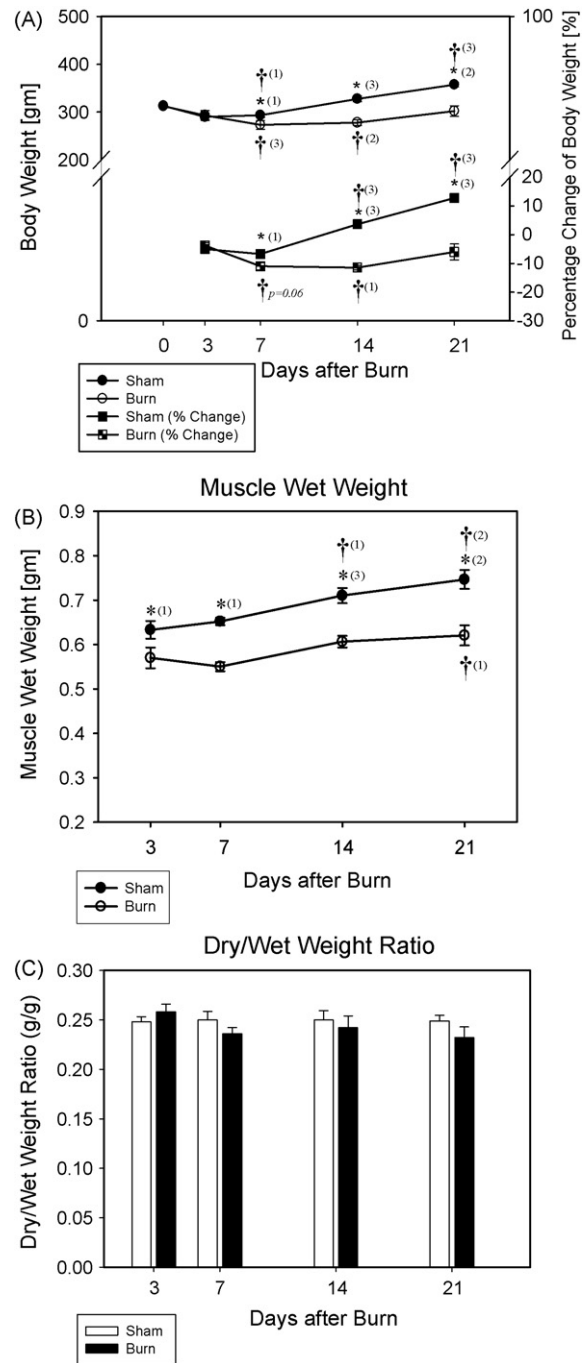


Fig. 1 – (A) Body weight/percentage change of body weight at days 3, 7, 14 and 21 after burn; circles represent body weight at days 0, 3, 7, 14, and 21; squares represent percent change in body weight at days 3, 7, 14 and 21. Body weight in burned rats was significantly lower than that of sham rats at days 7, 14 and 21. Significant differences were found in body weight percent change between groups at days 7, 14 and 21. (B) TA muscle wet weight at days 3, 7, 14 and 21 after burn: muscle wet weight in burned rats was significantly lower than that of the sham rats at days 3, 7, 14 and 21. (C) Muscle dry/wet weight ratio, no significant difference was found between groups. *Significant difference between the groups of burn and sham; †significant difference at days 7, 14 or 21 compared to day 3 respectively. ⁽¹⁾ $p < 0.05$; ⁽²⁾ $p < 0.01$; ⁽³⁾ $p < 0.001$.

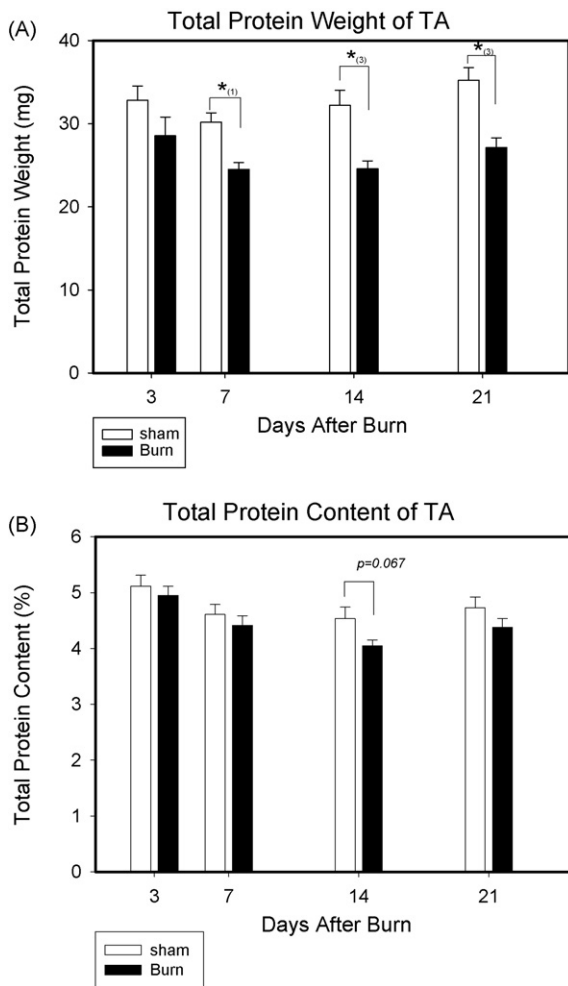


Fig. 2 – The total muscle protein weight and protein content percent of TA. (A) Total muscle protein weight: total muscle protein weight after burn was significantly lower than sham at days 7, 14 and 21. *Significant difference between the groups. ⁽¹⁾ $p < 0.05$; ⁽³⁾ $p < 0.001$. (B) Total muscle protein content percent: no significant difference of total muscle protein content percent between groups was found. * $p = 0.067$ comparison between burn and sham at day 14.

compared to sham at day 21 (Table 1). This may imply a higher percentage of slow-twitch fiber content in TA of burn than sham at this late time point.

The absolute Po progressively decreased in burned rats from day 3 to day 14 after injury with a significant difference between sham and burn at day 14 (burn: 10.64 ± 0.88 N and vs. sham: 14.56 ± 0.22 N, $p < 0.01$) (Table 1). Compared to the absolute Po of burn to sham, the percent reduction was -4.5% , -8.4% , -26.2% , and -5.5% at days 3, 7, 14, and 21 respectively. The absolute Po was 18.0% increased in shams, but was -9.8% decreased compared to the initial level respectively at day 3 when the biggest deficient was found between burn and sham. Similar to the absolute Po, the specific Po (proportion of Po to TA muscle weight) was also significantly lower in the burn group compared to sham at day 14 (burn: 16.49 ± 1.02 N/g vs. sham: 19.22 ± 0.17 N/g, $p < 0.01$) (Table 1). This suggests that the muscle strength of TA was not only proportionally reduced

by loss of muscle mass, but was also affected by the burn itself. Both absolute and specific Po recovered following burn at day 21 with no significant difference to sham at that time (Table 1). As a result of the increase in Pt, the Pt/Po ratio was significantly higher in the burn group at days 14 and 21. In addition, a significant correlation between tetanic tension and muscle weight was found ($r = 0.72$ and $r^2 = 0.51$, $p < 0.001$) (Fig. 3).

Fatigue index was not significantly different between groups (Table 2).

4. Discussion

Using an established model of hypermetabolism after severe burn in rats, we found a continuous loss of body weight with a peak low appearing between 7 and 14 days after injury with an associated loss of muscle mass in individual hind-limb muscles. As expected, this loss of muscle mass resulted in a reduction of Po (absolute). However, the novel finding of the study was that the loss of absolute Po was also associated with reduction in specific Po (N/g of muscle). Thus, the loss of peak tension was greater proportionally than can be accounted simply by loss of muscle mass. Therefore, we show here for the first time a decreased in strength per muscle mass unit after severe burn, indicating an associated intrinsic loss of muscle function over and above that associated with simple muscle mass loss.

Burn induces a hypermetabolic response and causes sustained negative net protein balance of skeletal muscle in human subjects up to almost 1 year after injury even though the wounds had healed [2]. Animal studies have also shown apoptotic loss of myofibrils in response to burn [23]. In this rat model, similar to the burned subjects, we found that muscle protein weight was significantly decreased after burn compared to sham, suggesting burn induced muscle protein catabolism. However, the muscle tetanic force of burn animals recovered to values near the sham animals at day 21 before the burn wound was completely healed. A likely explanation is that burned rats in this study were not restricted in movement after injury, and appeared to display normal cage activity. The attenuating affect of muscular activity on protein sparing, even during starvation has been well established [24]. In contrast, patients with severe burn are often undergoing long-term bed rest in the hospital, and have limited mobility once leaving the hospital while convalescing. Although exercise has been recommended to patients during their rehabilitation period, it is not clear whether long-term bed rest and inactivity also contribute to muscle catabolism seen after severe injury, and may play a role in delaying or prolonging the process of recovery for muscle mass or muscle strength.

Sham animals also had a drop in body weight and muscle weight (unlike no intervention controls, data not shown), which could have been due to responses caused by the stress of anesthesia. This was a temporary response, however, as usually within 3 days the animals quickly returned to normal growth patterns. In burned rats, body weight and muscle weight continuously decreased after 7 days, and remained below initial values until day 14 (muscle weight) and day 21 (body weight).

The predominant reason for burn related loss of force production was the loss of muscle mass as indicated by the

Table 1 – Muscle contractile properties (twitch tension). All values are mean $\pm$ SEM. Pt: twitch tension; Pt/MW: ratio of twitch tension/muscle weight; TPT: time to the peak tension; RT_{1/2}: half time to complete relaxation; Po: tetanic tension; Po/MW: ratio of titanic tension vs. muscle weight; Pt/Po: twitch-tetanic ratio.

	Day 3		Day 7		Day 14		Day 21	
	Sham	Burn	Sham	Burn	Sham	Burn	Sham	Burn
Pt (N)	4.29 $\pm$ 0.26	4.16 $\pm$ 0.33	4.33 $\pm$ 0.33	3.95 $\pm$ 0.26	4.75 $\pm$ 0.32	4.29 $\pm$ 0.43	3.94 $\pm$ 0.22	5.26 $\pm$ 0.5 ^{(2),†(1)}
Pt/MW (N/g)	7.04 $\pm$ 0.57	6.38 $\pm$ 0.43	6.50 $\pm$ 0.49	6.27 $\pm$ 0.36	6.27 $\pm$ 0.39	6.70 $\pm$ 0.61	5.26 $\pm$ 0.83	7.17 $\pm$ 0.93 ⁽²⁾
TPT (ms)	22.0 $\pm$ 0.8	22.4 $\pm$ 0.9	19.8 $\pm$ 0.8	21.0 $\pm$ 0.8	20.5 $\pm$ 0.7	20.0 $\pm$ 0.8	19.5 $\pm$ 0.7	22.4 $\pm$ 0.8 ⁽¹⁾
RT _{1/2} (ms)	16.8 $\pm$ 1.1	16.0 $\pm$ 1.2	14.9 $\pm$ 1.1	17.2 $\pm$ 1.0	14.5 $\pm$ 1.0	15.5 $\pm$ 1.0	14.0 $\pm$ 0.9	17.0 $\pm$ 1.1 ⁽¹⁾
Po (N)	12.34 $\pm$ 0.58	11.79 $\pm$ 0.64	12.29 $\pm$ 0.59	11.26 $\pm$ 0.54	14.56 $\pm$ 0.50 ^{Δ(1),†(1)}	10.64 $\pm$ 0.54 ^{*(3),†(1)}	13.39 $\pm$ 0.50	12.65 $\pm$ 0.58
Po/MW (N/g)	17.73 $\pm$ 0.67	19.99 $\pm$ 0.37	18.42 $\pm$ 0.16	17.94 $\pm$ 0.48	19.22 $\pm$ 0.17	16.49 $\pm$ 1.02 ^{(2),Δ(3)}	17.55 $\pm$ 1.07	18.10 $\pm$ 0.51
Pt-Po ratio	0.350 $\pm$ 0.03	0.353 $\pm$ 0.03	0.353 $\pm$ 0.03	0.353 $\pm$ 0.03	0.326 $\pm$ 0.02	0.409 $\pm$ 0.03 ^{*(1)}	0.297 $\pm$ 0.02	0.411 $\pm$ 0.03 ^{*(2)}

⁽¹⁾ $p < 0.05$; ⁽²⁾ $p < 0.01$; ⁽³⁾ $p < 0.001$.

* Significant difference between burn and sham at the same time point.

† Significant difference between day 14 and day 21.

‡ Significant difference between day 14 and day 7.

Δ Significant difference between day 14 and day 3.

linear regression between muscle weight and Po ($r = 0.71$, $r^2 = 0.51$, $p < 0.001$). However the greater loss in Po relative to the reduction in muscle mass suggests specific tension of muscle and/or neural input to muscle is reduced. Interestingly hind-limb unloading in rats [25,26] and bed rest in humans [27] has also been shown to result in a reduction in specific tension. This has been attributed to relative reduction in contractile proteins per individual fiber, resulting in a reduction in available cross-bridges. In this study, we found that a decrease in protein content percent per muscle mass in burn compared sham which peaked at 11% reduction in burn compared to sham at day 14. This may have contributed to the significant reduction in specific force in the burn group at that time. Future studies will determine whether the reduction of specific force is associated with loss of muscle motor protein without proportional muscle protein loss after burn. In addition, burn has recently been shown to exert a systemic effect on peripheral nerves [28,29]. While these studies only measured conduction velocity, it is possible that other aspects of the nerve-muscle interaction may be affected.

In this standard burn model, it was reported that only fast muscle fibers are affected and slow muscle fibers were mostly preserved [30–32]. We found similar results in that the muscle weight decreases were only shown in predominantly fast-twitch muscles such as TA, plantaris, extensor digitorum longus and gastrocnemius, and minimal change was found in predominantly slow-twitch muscles such as soleus (data not shown). In rats, the TA contains predominantly fast-twitch fibers (type IIb) [33,34], but is mixed with about 15% type I and type IIa fibers. We found that TPT, RT, and Pt/Po were increased in burn compared to sham at day 21, suggesting a potential fiber type shifting from fast to slow in TA after burn. It is not fully understood why twitch tension (Pt) was significantly higher in burn groups at day 21 compared to the groups of sham. The likely explanation is that burn induces a massive increase of Ca^{2+} influx in skeletal muscle [35,36], which may be associated with increased twitch

tension after burn, however, whether this is the cause of the change of sarcoplasmic reticulum Ca^{2+} uptake and Ca^{2+} -ATPase activity and then increase twitch tension was not determined in this study.

Muscle fatigue is defined as muscle failure to maintain expected force output. The cause of fatigue is a complex process which involves multiple factors. The relative importance of an individual factor is dependent on the fiber type composition of the contracting muscles. Slow-twitch fibers, including type I and fast oxidative glycolytic type IIa fibers, have high mitochondrial content and thus are relatively fatigue resistant compared to the fast glycolytic fibers [37]. In this study, although there was a temporarily increase in fatigue index of the TA at days 3 and 7 in both burn and sham,

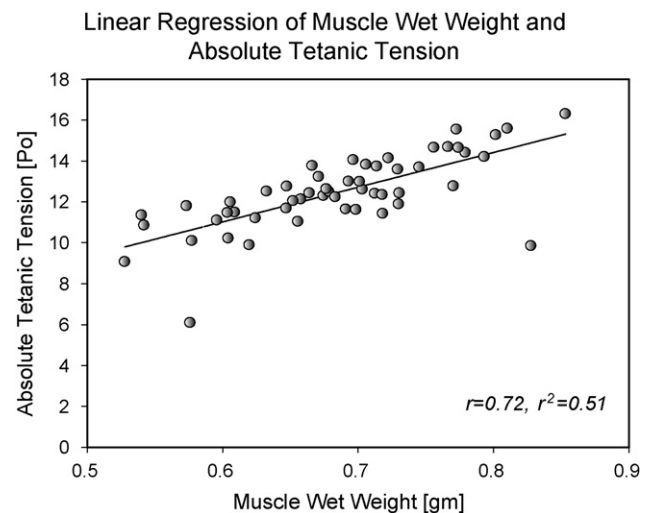


Fig. 3 – Linear regression between muscle wet weight and absolute tetanic tension. There was a linear regression between muscle wet weight and absolute tetanic tension. $r = 0.72$, $r^2 = 0.51$, and $p < 0.001$.

Table 2 – Fatigue index. All values are mean $\pm$ SEM. No significant difference was found between the groups.

	Day 3		Day 7		Day 14		Day 21	
	Sham	Burn	Sham	Burn	Sham	Burn	Sham	Burn
Fatigue index	0.171 $\pm$ 0.015	0.164 $\pm$ 0.014	0.196 $\pm$ 0.037	0.207 $\pm$ 0.013	0.130 $\pm$ 0.006	0.163 $\pm$ 0.014	0.151 $\pm$ 0.011	0.152 $\pm$ 0.009

the levels were both back to normal at day 21, and no significant difference was seen between groups. As discussed previously, the contractile property results suggested a potential fiber type shifting from fast to slow at day 21 after burn, so we might have expected to see some fatigue resistance increase at day 21 in response to burn. However, the fatigue index of burn returned to normal levels at day 21, suggesting a potential fiber type shift only from type IIb (fast oxidative glycolytic) to type I (slow oxidative) without changing the relative amount of fatigue resistant fibers (type IIa + type I) while decreasing the total number of fast-twitch fibers (type IIa + type IIb). This could explain why muscle contraction was slower than sham, but fatigue resistance was unchanged.

In conclusion, we demonstrated that the muscle catabolism decreases muscle strength and is initiated early after burn. These decreases in strength appear to be related not only to loss of muscle mass but also to decreases in general muscle fiber strength related to the injury. Resolution occurs in the first 3 weeks of injury, and seems to be related to a switch in fiber type from fast twitch to slow twitch.

Conflict of interest statement

Xiaowu Wu, Dr. Steven E. Wolf, and Dr. Thomas J. Walters have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript.

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