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14. ABSTRACT

Severe injury in patients results in adverse physiologic and musculoskeletal changes that are immediate and long lasting. In response to injury, metabolic and physiologic responses determine length of hospitalization and subsequent activity limitations. Reduced mobility from bed rest and injury severity affect muscle and bone health and are detrimental to rehabilitative success. Therefore, means to counteract adverse effects on muscle and bone after injury and disuse are needed. Pharmacologic (i.e insulin or oxandrolone), non-pharmacologic (i.e. exercise) and nutritional interventions have been used independently with limited success [17]. The combination of pharmacological interventions and exercise has not been systematically investigated. **We propose to determine if the administration of pharmacologic agents combined with exercise attenuates muscle atrophy and bone degradation following severe injury, disuse and re-ambulation. The current proposal is for mitigation of musculoskeletal pathologic change in polytrauma patients.**

15. SUBJECT TERMS
Nothing listed

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1. INTRODUCTION

Background: Severe injury results in physiologic and musc9uloskeletal changes to the patient that
are immediate and long lasting. Reduced mobility from bed rest and injury severity affect muscle and bone health and are detrimental to rehabilitative success. We propose to determine if the combination of exercise and the use of insulin or oxandrolone will further improve muscle and bone strength and subsequent function for improved quality of life. The specific aims of this study are to: 1) Characterize the effect of resistance exercise on muscle and bone health in a validated model of burn and disuse. 2) Evaluate the effect of resistance exercise in combination with currently used pharmacological therapies (insulin or oxandrolone) on muscle and bone health in a validated model of burn and disuse. 3) Determine the interrelationship between muscle and bone after re-ambulation following pharmacological interventions and exercise. To accomplish these aims we will use our established rat model of burn and disuse for a 14 day period. Rats will be assigned to vehicle or drug treatment and will be further randomized into either exercise or no exercise groups. After 14 days, additional studies will examine the effects of re-ambulation Analysis will be completed on blood, tissues, and bones of the study animals. This proposed project will delineate the synergistic effects of current therapy that will be directly translational to the clinical care of military polytrauma victims.

2. KEYWORDS

- Rats
- Burn
- Hindlimb Unloading
- Exercise
- Disuse
- Re-ambulation

3. OVERAL PROJECT SUMMARY

Specific Aim 1 was to characterize the effect of resistance exercise on muscle and bone health in a validated model of burn and disuse. The milestones for Year 1 of the project, which were completed, included beginning and completing experiments for Aim 1, including animal, assay work, data analysis, and presentation. There were eight major tasks associated with this milestone.
Specific Aim 2 was to evaluate the effect of resistance exercise in combination with currently used pharmacological therapies (insulin or oxandrolone) on muscle and bone health in a validated model of burn and disuse. The milestone for Year 2 of the project was to complete experiments for Aim 2, including animal assay work and data analysis. There were six major tasks associated with this milestone.
Specific Aim 3 was to investigate the role of reloading and resistance exercise in the animal model, The experiment schedule for Specific Aim 3 was approved by DOD for one year extension due to major personal translocation in Year 3. The milestone for Year 4 was to complete experiments for Aim 3, including animal assay work and data analysis. There were eight major tasks associated with this milestone.

RESULTS SUMMARY (WOLF):

FOR SPECIFIC AIM 1:

- The reversal effect of exercise training on muscle genomic profile in rats with burn and hind limb

Micro RNA (miRNA) is a class of non-coding RNA that regulates gene expression by silencing messenger RNA. We pooled 3 animal muscle samples from each treatment group for RNA extraction. We measured miRNA expression by using Affymetrix miRNA 4.0 Arrays and gene expression by using Affymetrix rat gene 2.0 chips.

MiRNAs and gene profiles are distinguished in response to burn, hindlimb unloading and exercise respectively. There are 1,218 rat spice miRNAs in a total 36,222 miRNAs detected in each group. We identified 703 (57.7%) up-regulated miRNAs and 515 (42.3%) down-regulated miRNAs in the burn group compared to sham. 35 up-regulated and 12 down-regulated genes after burn in rat plantaris. 623 miRNAs were upregulated and 587 were down regulated with exercise.41 gene transcript probes were identified including 40 down-regulated and 1 up-regulated between the exercise or non-exercise in BH rats.

Burn and hind limb unloading contribute respectively. However, there are overlaps in both miRNA and transcript gene levels between burn and hindlimb unloading. MiR-182 increased 12.81 fold in burn, 23.82 fold in hindlimb unloading respectively, and increased 35.35 fold in burn and hindlimb unloading group. Nr4a3 gene expression increased 2.45 in burn and 3.59 fold in hindlimb unloading respectively, and increased 6.31 fold in BH rats.

One targeted gene could be regulated by a group of miRNAs. For instance, miR-409a-3p was the most down-regulated miRNA in response to burn (-2.95 fold change). It functions with up-regulated miRNA-182 to inhibit muscle Col1a2 gene expression after burn. Genes related pathways are activated after burn, such as inflammation response, oxidative stress, cell cycle, cell apoptosis, calcium regulation, striated muscle contraction.

The effect of exercise alleviated miRNA and gene expression in BH rats. miR-182 decreased -7.04 fold in BH rats with exercise training; gene expressions of Fgl2 in blood clotting cascade and Col1a1 in inflammatory response pathway decreased in response to exercise training as well.

In summary, miRNAs and transcript gene profiles were affected in burn and hindlimb unloading, those changes are associated with muscle pathophysiological changes, including muscle mass loss and function impairment. The muscle improvement with exercise training were also observed in gene levels with miRNA alterations.

FOR SPECIFIC AIM 2:

- The improvement of exercise and insulin pharmacological combination in rat muscle

Twenty Four animals received burn and hindlimb unloading procedure (as previous experiment) and were randomly assigned (n=6) to vehicle without exercise (V/N), insulin (pro zinc 40U daily) without exercise (I/N), vehicle with exercise (V/E), or insulin with exercise (I/E). On day 14 muscle functions were tested and tissue collected.

In summary, we observed that muscle functions including tetanic (Po) and twitch (Pt) were significantly elevated in both plantaris and soleus with insulin and excise combined treatment. No function improvement with solely insulin treatment was found at the moment. (Data values presented as mean ±SEM listed the following table)

Muscle Dimensions and Isometric Muscle Function

Parameter		Plantaris				Soleus			
Group		No Exercise		Exercise		No Exercise		Exercise	
		Vehicle	Insulin	Vehicle	Insulin	Vehicle	Insulin	Vehicle	Insulin
Muscle	Wet weight (mg)	332 ± 18	329 ± 9	348 ± 6.3	354 ± 14	119 ± 2	143 ± 23	151 ± 32	131 ± 7
	Lo (mm)	35 ± 2	31 ± 0.3	32 ± 1	32 ± 1	33 ± 1.4	29 ± 0.4	31 ± 0.9	30 ± 0.4
	PCSA (mm^2)	27 ± 3	30 ± 2	30 ± 2	#31 ± 2	5.0 ± 0.2	6.9 ± 2.3	6.7 ± 1.3	6.0 ± 0.3
Twitch Force	Pt (g)	89 ± 9	85 ± 3	92 ± 2	‡102 ± 8	10 ± 2	10 ± 2	14 ± 2	*18 ± 1
Tetanic Force	Po (g)	430 ± 31	459 ± 12	508 ± 14	†522 ± 17	38 ± 8	38 ± 9	59 ± 5	*69 ± 5
	Po/CSA (N/cm^2)	16 ± 2	15 ± 2	16 ± 1	17 ± 1	7.4 ± 2	7.0 ± 1	10 ± 2	‡12 ± 1
Pt/Po (%)		21 ± 1	19 ± 1	18 ± 0.4	20 ± 1	26 ± 1	24 ± 2	24 ± 2	26 ± 2
Fatigue	Maximum (g)	——	——	——	——	33 ± 6	34 ± 9	53 ± 4	*64 ± 4
	Minimum (g)	——	——	——	——	27 ± 6	27 ± 6	†46 ± 4	*54 ± 4
	Index (%)	——	——	——	——	81 ± 7	84 ± 5	87 ± 5	84 ± 5

Lo = optimal muscle length
PCSA = Physiological Cross Sectional Area
Po/CSA = Tetanic force normalized to PCSA
Pt/Po (%) = Ratio of twitch to tetanic force
Fatigue Index = Ratio of fatigue minimum to maximum

* vs. No Exercise (ANOVA, $p<0.05$)
† vs. Vehicle No Exercise (ANOVA, $p<0.05$)
# vs. Vehicle No Exercise (one-tailed t-test $p<0.05$)
‡ vs. Other groups combined (two-tailed t-test, $p=0.05$)

Signal regulation pathway study revealed that both insulin treatment and exercise training increased PDK/Akt protein synthesis pathway and decreased protein degradation pathway by reduction of muscle ring-finger protein-1(MuRF-1), an E3 ubiquitin ligase expression.

- <u>The effect of exercise and oxandrolone pharmacological combination in rat muscle function</u>

Twenty Four animals received burn and hindlimb unloading procedure (as previous experiment) and were randomly assigned (n=6) to vehicle without exercise (V/N), oxandrolone (daily) without exercise (O/N), vehicle with exercise (V/E), or oxandrolone with exercise (O/E). On day 14 muscle functions were tested and tissue collected.

Two way ANOVA statistical analysis showed that there is significant decreased fatigue index (FI) with exercise training, like we observed in previous study. There is no significant changes with oxandrolone treatment under the current analysis. (Data values presented as mean ±SD listed the following table)

Plantaris	NX		EX		Soleus	NX		EX	
	VEH	OXD	VEH	OXD		VEH	OXD	VEH	OXD
Tissue weight(g)	0.330 ± 0.018	0.312 ± 0.028	0.331 ± 0.042	0.300 ± 0.033	Tissue weight(g)	0.109 ± 0.015	0.100 ± 0.031	0.102 ± 0.014	0.110 ± 0.016
Lo(mm)	22.2 ± 1.8	22.2 ± 2.5	22.3 ± 1.1	22.3 ± 1.2	Lo(mm)	21.8 ± 2.4	22.0 ± 3.0	21.4 ± 3.4	20.8 ± 0.8
1/2 RT(s)	0.0166 ± 0.001	0.0169 ± 0.001	0.0187 ± 0.001	0.0165 ± 0.001	Pt(g)	47.80 ± 29.44	52.30 ± 14.13	64.95 ± 39.77	48.82 ± 11.19
Pt(g)	98.00 ± 13.39	115.85 ± 27.13	112.40 ± 22.47	105.86 ± 11.51	Po(g)	113.49 ± 69.89	126.02 ± 49.97	106.30 ± 18.62	124.09 ± 17.81
Po(g)	426.67 ± 137.64	469.08 ± 47.16	489.74 ± 58.13	555.44 ± 45.41	FI	26.43 ± 11.79%	27.24 ± 13.02%	15.56 ± 7.75%	16.08 ± 6.36%
Po/Pt	4.29 ± 1.01	4.17 ± 0.70	4.48 ± 0.94	5.31 ± 0.55	Fatigue (max)(g)	79.89 ± 45.63	95.075 ± 36.44	100.25 ± 13.55	104.71 ± 11.34
sPt(N/cm2)	6.787 ± 0.804	8.969 ± 2.974	7.853 ± 1.449	8.187 ± 1.020	sPt(N/cm2)	9.693 ± 5.549	10.814 ± 4.504	14.055 ± 8.328	9.521 ± 1.741
sPo(N/cm2)	29.686 ± 10.058	35.212 ± 7.434	34.733 ± 6.979	38.251 ± 11.784	sPo(N/cm2)	22.671 ± 14.044	29.082 ± 13.787	23.413 ± 6.091	24.459 ± 4.136

FOR SPECIFIC AIM 3:

Twenty Four rats received 40% TBSA) burn and hindlimb unloading (HLU) described previously. All animals received exercise training for 14 days; meanwhile half of them were treated with pro zinc insulin 5U/kg subcutaneous daily and half with vehicle saline injection. Reloading period starts on day 14. All rats were removed from HLU and all injections stopped. Rats within each treatment group were separated into exercise (EX)/no exercise (NEX) (n=6) for another 14 days.

On day 28 *in situ* isometric forces of the left soleus and plantaris muscles were examined. The isometric twitch tension Force (Pt) and muscle optimal length (Lo) decreased in left plantaris from rats with exercise treatment significantly. Not like fast twitch dominated plantaris, rat slow-twitch myofiber dominated soleus tetanic force (Po) with insulin treatment increased significantly.

4 KEY RESEARCH ACCOMPLISHMENTS

- The milestone for Year 4 was the completion of Aim 3. There were 8 major tasks associated with this aim. See **Appendix 1** for project timeline. The tasks and completion status are included below:

- Major Task 1 was to obtain Animal Use Approval from the University of Tesas Health Science Center at Houston. This task was completed before any associated animal wrok could be completed (Wade).

- Major Tasks 2 and 3 included training personnel, acquiring any equipment needed for the experiments associated with Aim 3 and setting up for the animal testing. These tasks were *completed* prior to any work being done. Coordination with investigators from the other institutions was initiated and timelines were able to be generated experiment completion (Wade/Wolf).

- Major task 4 includes starting and completing Aim 3 experiments. All pre-training and experimental procedures were *completed* according to the timeline. Samples were collected and stored appropriately for further analysis (Wade/Wolf).

- Major task 5 was *completed* at the conclusion of animal experiment. Muscle function was completed on all animals in all groups for Aim 3. Plasma and tissue were collected and stored for processing at a later time (Wade). Muscle function testing was completed and muscle sample was stored (Wolf).

- Major task 6: Sample Analysis is currently *on-going*.
    - Subtask 1: Order ELISAs - *See Partnering PI Technical Report*
    - Subtask 2: Complete ELISAs - *See Partnering PI Technical Report*
    - Subtask 3: Complete muscle tissue weight measurement—*completed (Wolf)*
    - Subtask 4: Complete muscle RNA/protein extraction - - *ongoing (Wolf)*
    - Subtask 5: Complete histology & immunohistochemistry staining - *ongoing (Wolf)*
    - Subtask 6: Complete muscle tissue protein electrophoresis- *Pending (Wolf)*
    - Subtask 7: Genomic profile analysis – *ongoing (Wolf)*

- Major Task 7: Bone Analysis is currently *on-going*. (*See Partnering PI Technical Report)*

- Major Task 8: Final Aim III Data Analysis is currently *on-going*. 5 abstracts have been submitted to professional scientific meetings detailing this work to-date. 2 manuscripts has been accepted/published in a peer-reviewed journal detailing this work to-date.

5 CONCLUSION

Both Aim 1 and Aim 2 used our validated rodent model of burn and disuse, with a daily resistance exercise regimen started before injury and continued for the duration of the experimental period. Aim 2 focused on the addition of daily doses of either vehicle or a pharmacological agents (insulin or oxandrolone) in unison with the daily resistance exercise regimen. For both aims, all rats were able to complete the exercise program after injury and no rats were excluded from the experiment at any time. Data presented, irrespective of resistance exercise and daily dosing, are comparable to previous studies. Daily resistance exercise resulted in a significant change in body mass, which can be attributed to the reduction in fat mass. The changes in muscle and bone support our previous research. Bone was affected primarily by the disuse component. Exercise alone did not seem to contribute to the overall changes. Muscle changes, however, were a result of the addition of resistance exercise. Transcriptomic gene profiles are comparable reflected to skeletal muscular pathophysiological changes in response to treatments. All eight major tasks for Year 1 have been completed. All six major tasks for Year 2 have been completed or are currently on-going.

Animal experiment and muscle isometric force test for Aim 3 are completed. The primary focus is to determine the inter-relationship between muscle and bone following re-ambulation following pharmacological interventions and exercise. Muscle function data implied that myofiber type affect the response of exercise. All eight major tasks for Year 4 have been completed or are currently on-going.

6 PUBLICATIONS, ABSTRACTS AND PRSENTATIONS

(Abstract/Manuscripts (Appendix 2):

ABSTRACT PRESENTED AND SUBMITTED IN 2017: (2016 Sep to 2017 Aug)

2017- ORS 47th International Musculoskeletal Biology Workshop PRESENTED *(Abstract content attached)*

1. Paula Hernandez, Dustin Buller, Thomas Mitchell, Jamie Wright, Haixiang Liang, Kshitij Manchanda, Tre Welch, Ryan M. Huebinger, Deborah L. Carlson, Steven E. Wolf, Juquan Song. **Inflammation and Remodeling of Rat Achilles Tendon after Severe Burn.** ORS 47th International Musculoskeletal Biology Workshop at Sun Valley in beautiful Sun Valley, Idaho, August 6 – 9, 2017

2017- Shock Society Annual Meeting PRESENTED *(Abstract content attached)*

1. Juquan Song, Lisa Baer, Charles E Wade, Steven E Wolf. **Transcriptomic profile alterations in burn/hindlimb unloaded rats with insulin and exercise combination treatment.**

2018- ABA Annual Meeting SUBMITTED *(Abstract content attached)*

1. Calvin Geng, Nishika Karbhari, Juquan Song, Lisa Baer, Charles Wade, Steven E.Wolf, **Insulin and exercise combination therapy recovers muscle function in a burn and disuse rat model by activating protein synthesis and inhibiting**

2. Juquan Song, Kevin DeSpain, Lisa Baer, Charles E Wade, Steven E Wolf. **A long-term of resistant exercise decreased rat muscle function in fast twitch myofiber dominated plantaris**

3. Paula Hernandez, Andrea Fa, Thomas Mitchell, Dustin Buller, Ryan Huebinger, Michael Van Hal, Steven E. Wolf and Juquan Song. **Molecular and structural changes in Intervertebral Discs following Severe Burn in Rats.**

MANUSCRIPT PUBLISHED /ACCPETED:

1. **Song J**, Saeman MR, Baer LA, Cai AR, Wade CE, **Wolf SE**. Exercise Altered the Skeletal Muscle MicroRNAs and Gene Expression Profiles in Burn Rats With Hindlimb Unloading. J Burn Care Res. 2017 Jan/Feb;38(1):11-19. doi: 10.1097/BCR.0000000000000444. PMID:27753701

2. Paula Hernandez, Dustin Buller, Thomas Mitchell, Jamie Wright, Haixiang Liang, Kshitij Manchanda, Tre Welch, Ryan M. Huebinger, Deborah L. Carlson, Steven E. Wolf, Juquan Song. **SEVERE BURN-INDUCED INFLAMMATION AND REMODELING OF ACHILLES TENDON IN A RAT MODEL**. SHOCK. 2017 October, Accepted

(Manuscript attached separately)

7. INVENTIONS, PATENTS and LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

Nothing to report.

9. OTHER ACHIEVEMENTS

Nothing to report.

10. REFERENCES

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rats. Am J Physiol 1994;267: R365-71.
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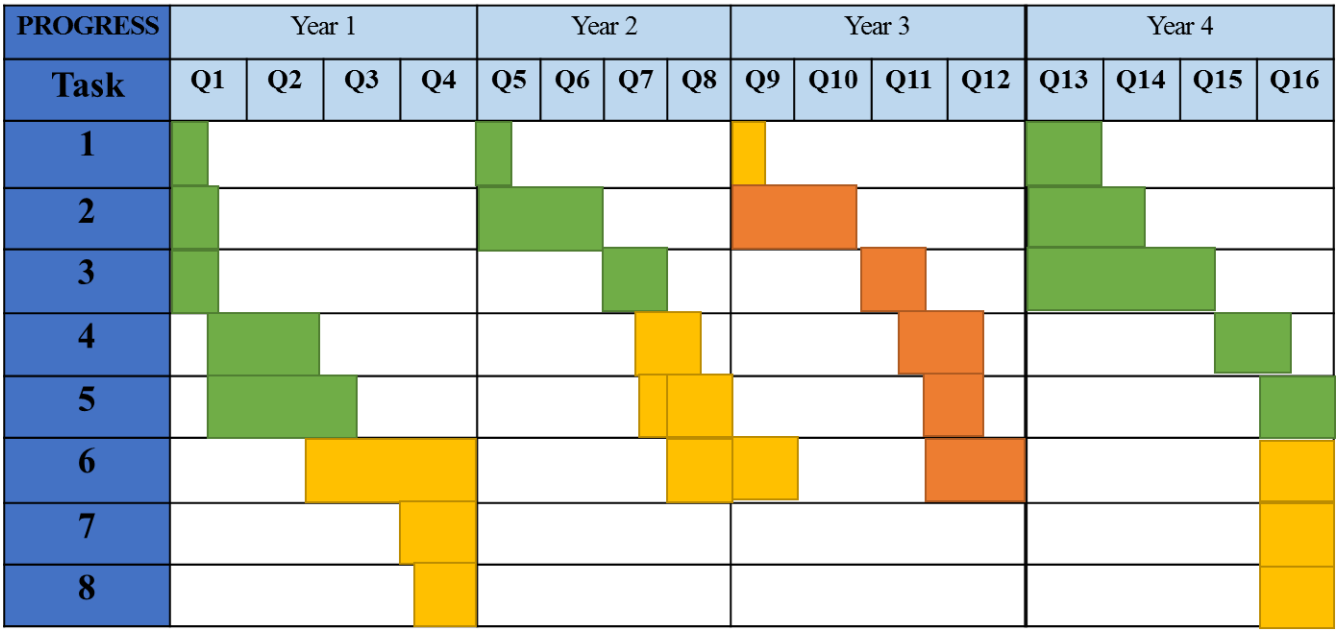
Contributions of severe burn and disuse to bone structure and strength in rats. Epub 2012 Nov 7.
10. Pidcoke HF, Baer LA, Wu X, Wolf SE, Aden JK, Wade CE**.** Insulin effects on glucose tolerance, hypermetabolic response, and circadian-metabolic protein expression

11. APPENDICES

1. Project Timeline
2. Submitted Abstracts
3. Published/Accepted Manuscripts (separate attached files)
4. Quad Chart (separate attached file)

Project Timeline **-TASK PROGRESS SUMMARY CHART**



Completed

On-Going

Planned

Wolf 2017 annual

APPENDIX - PUBLICATIONS, ABSTRACTS AND PRSENTATIONS

ABSTRACT CONTENT:

ORS 47th International Musculoskeletal Biology Workshop

Inflammation and Remodeling of Rat Achilles Tendon after Severe Burn

Paula Hernandez[1]; Dustin Buller[1];Thomas Mitchell[1]; Jamie Wright[2]; Haixiang Liang[4]; Kshitij Manchanda[1]; Tre Welch[2]; Ryan M. Huebinger[3]; Deborah L. Carlson[3]; Steven E. Wolf[3]; Juquan Song[3]*

[1]Department of Orthopaedic Surgery, [2]Department of Cardiovascular and Thoracic Surgery, [3]Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX; [4]Orthopedic Research Laboratory, Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY.

INTRODUCTION: Severe burn, a full-thickness burn of greater than 30% of total body surface area (TBSA), may affect multiple organs, even those distantly located from the burn site. In the musculoskeletal system, studies have focused mainly on muscle and bone, where hypercatabolism due to the activation of systemic inflammation, and disuse from long immobilization periods is induced. Muscle loss and atrophy post burn have been associated with increased levels of TNF-α, while bone mass loss has been associated with circulating IL-1β and IL-6, which are increased from 24 hours post burn. We aim to study the molecular and structural effects of burn injury on tendon in an effort to ultimately understand the behavior of the whole muscle- tendon-bone organ. We report acute local molecular and functional changes in Achilles tendon as result of severe burn-induced systemic inflammation.
METHODS: *Severe burn injury:* 40 adult male Sprague-Dawley rats, 270-300 g, were used with protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern. Animals were randomly divided into 5 groups: Control (n = 11), 1 day (n = 6), 3 days (n = 6), 7 days (n = 6), and 14 days (n = 11) post burn. Under anesthesia, animal hair was removed from the dorsal and lateral surfaces. Dorsal skin amounting to 40% of TBSA was immersed in 95°C-100°C water for 10 seconds. Control animals received sham treatment. *Gene and protein expression*: Tendon RNA was extracted and analyzed for expression of IL-6, TNF, IL-1β, TGFβ1, col1a1, col3a1, MMP9 and MMP13 by qPCR. 10 µg of total protein was used for Western blot with anti-collagen I (Abcam), anti-collagen III (Abcam). *Histology*: After tendon fixation and decalcification, paraffin sections were processed for H&E. *Biomechanics:* Testing was done in an Instron 5565 universal testing system equipped with a 5 kN load cell. Samples were pulled until failure at a cross-head speed of 6 mm/min collecting force and deformation data throughout the test. *Statistics*: Data are presented as mean ± error propagation for gene expression and mean ± standard deviation elsewhere. Data were analyzed in GraphPad Prism 7 with one-way ANOVA and Fisher's LSD posthoc test, or by unpaired Student's t test when comparing two variables, ($p < 0.05$ being significant).
RESULTS: Gene expression of IL-6 and IL-1β as well as MMP9 and MMP13 increased in rat tendon 3 days after burn. TNF did not reached significant difference. Moreover, TGFβ1 a marker for tissue repair, was elevated after 14 days. Protein expression of Collagen I decreased at day 7 and returned to control levels by day 14. Together with this, we observed a trend to increase of col1a1 gene expression at day 7 (p=0.055). On the other hand, col3a1 gene expression is significantly higher at day 3 post burn, while an increase in protein Collagen III was observed by day 14 post burn. Protein ratio for Collagens I/III decreased at day 14, $p < 0.05$, indicating signs of tendon remodeling. Histological analysis showed higher cell density in tendon periphery and surroundings at days 1 and 3 post burn. Biomechanical analysis showed a decrease in stiffness and ultimate force of tendons in burn rats after 14 days.
DISCUSSION: The upregulation of both IL-1β and IL-6 indicates the initiation of acute inflammation in the tendon. When tendon experiences remodeling or injury, collagen III content increases, resulting in a decrease in tensile strength. On the other hand, metalloproteinases such as the collagenase MMP13 can cleave the collagen triple helix, creating fragments that are further degraded by gelatinases such as MMP9. We propose that the initial decrease in collagen I can be explained by the upregulation of MMP13 and MMP9, generated by IL-1β. We hypothesize that this event is started by the cell infiltration observed in histological analysis. We are currently investigating the involvement of macrophages. The synthesis of collagen III protein, the decrease in collagen I/III protein ratio and the increase in TGFβ1 suggests an induction of scar tissue or remodeling. To address whether molecular changes in tissue structure will also affect its functional mechanics, the tensile force and deformation were measured. Overall, our findings showed that ultimate force and stiffness were reduced at 14 days post burn compared to controls. These biomechanical results correlate with histological findings of less organized fibers. Although previous reports have shown that Achilles tendon could react to an inflammatory event produced in surrounding tissue (*8*), this study is the first to report that remote systemic inflammation is capable of inducing a local inflammatory response and remodeling in the tendon.

2017- Shock Society Annual Meeting

Transcriptomic profile alterations in burn/hindlimb unloaded rats with insulin and exercise combination treatment

Juquan Song, Lisa Baer, Melody Saeman, Charles E Wade, Steven E Wolf
Department of Surgery, University of Texas Southwestern, Dallas, TX;

We previously demonstrated changes in miRNA related gene profiles in burn and hindlimb unloaded (B/H) rats. We also found that exercise training reversed those gene profiles which was principally related to diminished oxidative stress and inflammatory signaling. Insulin treatment has also shown positive effects in burns; we recently observed that insulin additively improved muscle function when combined with exercise treatment in burn and hindlimb unloaded rats. We wondered whether transcriptome profiles reflect skeletal muscle pathophysiological changes in this event.
Twenty-four Sprague-Dawley rats received a full thickness 40% total body surface area (TBSA) burn and placed in a tail traction system for hindlimb unloading to mimic bed rest immediately following burn. Half were trained to perform twice daily weighted resistance climbing of 1 meter with 5 repetitions, while the other half was not. Six rats the exercise and no exercise groups received insulin injection subcutaneously (5U/kg daily). On day 14 plantaris muscles were harvested and tissues were grouped for RNA extraction and genomic data analysis applied with Affymetrix Analysis Console 3.0 software.
With threshold for absolute fold change greater than 2, we found 59, 48 and 93 miRNAs altered in rat muscle with insulin, exercise, and combination treatment respectively; miR-499-5p was the most increased with 15 fold in the combination group. For gene expression, we found 122, 119 and 170 changes in insulin, exercise and combination treatment respectively. Act1, Sln, Tecrl were the most upregulated, and Mbp, Pmp2, Mpz were the most down-regulated genes with exercise and insulin treatment. Wikipathway analysis showed that striated muscle contraction pathways were improved with both exercise and insulin treatment, and directly correlated with the muscle function improvement. Meanwhile, TGF beta and TLR signaling pathways were inhibited with both treatments.
In conclusion, exercise and insulin contribute additively to miRNA and gene expression changes after burn and immobilization, and reflect physiologic muscular improvement. The finding of the study identifies the specific pathway signals affected by insulin and exercise.

External Funding: This project was funded by DOD grant #W81XWH-13-1-0489

2018- ABA Annual Meeting

Insulin and exercise combination therapy recovers muscle function in a burn and disuse rat model by activating protein synthesis and inhibiting proteolysis

Calvin Geng, Nishika Karbhari, Juquan Song, Lisa Baer, Charles Wade, Steven E. Wolf
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Introduction

Burn injuries bring about a hypermetabolic state that results in a loss of muscle mass and function. Like burns, disuse of muscle also results in muscle loss[2]. Resistance exercise and insulin have individually been shown to attenuate burn and disuse induced muscle atrophy, though neither is fully compensatory. To date, there is no data on the efficacy of insulin and exercise as a combination therapy to recover muscle mass and function. This project investigates the molecular mechanisms behind musculoskeletal pathophysiological improvements in a burn and disuse rat model given these treatments. Muscle function, protein synthesis/proteolysis pathway protein levels, and genomic profiles are examined.

Methods

24 Sprague-Dawley rats received full thickness 40% total body surface area burns and hindlimb unloading and were randomly grouped into vehicle without exercise (V/N), pro zinc 5U/kg of insulin without exercise (I/N), vehicle with exercise (V/E), and insulin with exercise (I/E) groups. 14 days after injury, hindlimb muscle function was measured and muscle tissues were harvested for genomic profile and western blot analysis.

Results

The isometric force including tetanic (Po) and twitch (Pt) were significantly elevated in the plantaris of I/E rats. The soleus also had significant elevation of Po, Pt, fatigue maximum, and fatigue minimum in I/E rats. Affymetrix transcriptome analysis determined that 70, 62, and 116 genes were upregulated more than 2 fold in insulin, exercise, and combination treatment, respectively. Western blots showed that p-PDK 1, which activates AKT activity, was significantly increased in all treatment groups compared to control ($p<0.01$). p-AKT S473 was significantly increased in the combination group ($p<0.05$). eEF2 controls the elongation step in translation and was increased in the exercise and combination. ($p<0.05$). Muscle RING-finger protein-1(MuRF-1), an E3 ubiquitin ligase, was reduced in the combination group ($p<0.05$).

Conclusions

Insulin and resistance exercise have a positive combined effect on muscle function recovery. Signal pathway examination showed that the combination treatment decreased protein degradation and increased protein synthesis. The observed changes at the transcriptional and protein levels are supported by muscle function improvements.

Applicability of Research to Practice

Muscle loss is a sequela of burn and disuse that increases cost and risk of complications. By identifying the molecular basis of these changes, treatments that target critical proteins can be developed to mitigate muscle loss and improve patient outcomes.

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A long-term of resistant exercise decreased rat muscle function in fast twitch myofiber dominated plantaris

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Abstract:
Introduction: We previously showed muscle function was impaired with hindlimb unloading in burned rats which was alleviated by insulin treatment and resistance exercise. In the current study, we investigated the role of continued resistance exercise to further improve function in a model designed to mimic the late recovery period akin to the rehabilitation phase in patients.
Methods: Twenty-four Sprague-Dawley rats received a full thickness 40% total body surface area (TBSA) burn and hindlimb unloading (HLU) to mimic severe burn with muscle disuse. All animals underwent exercise training twice a day with 5 climbs per training session. Resistance exercise was achieved by adding weight to the base of the tail and increased every 3 days. All rats were given a subcutaneous injection of either saline or pro zinc insulin 5U/kg daily. On day 14, all rats were removed from HLU and all injections stopped. Then, rats within each treatment group were separated into no exercise (NEX) and exercise (EX) groups (n=6 per group) for an additional 14 days. On day 28, *in situ* isometric forces of the left soleus and plantaris muscles were measured. Values are presented as mean ± SD. Statistical analysis was by two-way ANOVA.
Results: Plantaris isometric twitch tension force (Pt) and muscle optimal length (Lo) significantly decreased with exercise treatment for 28 days (149.40±16.95g NEX vs Pt: 131.99±17.84g EX) (p=0.028); (39.42±1.77mm NEX vs Lo: 37.08±1.99mm EX) (p=0.008). However, soleus tetanic force (Po) increased significantly in those treated with insulin previously with or without continued exercise (183.01±33.33g vehicle vs 220.61±26.01g insulin) (p=0.01). Further, the ratio of single twitch force to maximal tetanic force (Pt/Po) significantly decreased in the soleus with exercise treatment (0.32±0.08 NEX vs. 0.26±0.02 EX) (p=0.037).
Conclusions: In our previous study, we found the combination of insulin treatment and exercise after burn and hindlimb unloading improved muscle function in both plantaris and soleus. In the current study, the effect of previous insulin treatment further augments improvements at 28 days after injury in slow twitch muscle. However, continued resistance exercise actually decreased muscle isometric force in the fast twitch myofiber dominated plantaris with no change in the slow-twitch soleus. The decrease in Pt/Po ratio in slow-twitch myofiber dominated soleus suggests a myofiber type change in response to continued resistance exercise.
Applicability of Research to Practice: The current study provides evidence of appropriate type of exercise in burn patient rehabilitation.

Molecular and structural changes in Intervertebral Discs following Severe Burn in Rats

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INTRODUCTION:

Intervertebral discs (IVD) connect to the spinal vertebrae. IVD impairment and dysgenesis are clinically relevant to pain management and movement restriction. Severe burn disrupts skeletomuscular metabolic status. IVD response following severe burn is currently unknown. Transient receptor potential cation channel subfamily V member 4 (TRVP4) protein is a Ca^{2+}-permeable, nonselective cation channel which has been recently reported to be elevated in human disc degeneration. The aim of this study is to investigate the role of TRVP4 in rat IVDs following thermal injury.

METHODS:

Under a UTSW IACUC approved protocol, 40 adult male Sprague-Dawley rats were examined in this study. Animals received 40% of total body surface area (TBSA) scald burn with the standard procedure under anesthesia and randomly grouped: Control (n = 11), 1 day (n = 6), 3 days (n = 6), 7 days (n = 6), and 14 days (n = 11) post burn. Total RNA was extracted from whole IVDs and analyzed for expression of IL-6, TNF, IL-1β, MMP9, MMP13 and TRPV4 by qPCR. Lumbar IVD was also fixed for histological analysis. Data are presented as mean ± standard deviation. Data were analyzed in GraphPad Prism 7 with one-way ANOVA and Fisher's LSD posthoc test, or by unpaired Student's t test when comparing two variables, ($p < 0.05$ being significant).

RESULTS:

Gene expressions of IL-6, TNF and IL-1β were not altered in rat IVD after burn. ILMMP9 and MMP13 gene expression showed a significant upregulation in thoracic IVD at day 1 after burn. Histological analysis of lumbar IVD showed an increase in nucleus pulposus (NP) height in discs at days 1 and 3 after burn compared to control discs, indicating tissue swelling following thermal injury. The gene expression of the calcium-permeant channel TRPV4, activated by osmotic changes, showed a significant upregulation in both thoracic and lumbar IVD at day 3 after burn.

SMMARY:

Local response of IVD was observed with the increased height of NP and the increased gene expression of MMP9 and MMP13. The elevation of TRPV4 gene expression after burn indicates local mechanical/osmotic changes in IVDs. Future investigations will focus on the acute structural changes and if these alterations lead to late degeneration of the IVDs in the current animal model.

Clinical relevant: Patients start experiencing back pain and disc degeneration long after trauma occurs, it is crucial to understand the early events occurring at the cellular level in IVD triggered by trauma.

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Exercise Altered the Skeletal Muscle MicroRNAs and Gene Expression Profiles in Burn Rats With Hindlimb Unloading

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This study investigated microRNA and target gene profiles under different conditions of burn, bed rest, and exercise training. Male Sprague-Dawley rats (n = 48) were assigned to sham ambulatory, sham hindlimb unloading, burn ambulatory, or burn plus hindlimb unloading groups. Rats received a 40% TBSA scald burn or sham treatments and were ambulatory or hindlimb unloaded. Rats were further assigned to exercise or no exercise. Plantaris tissues were harvested on day 14 and pooled to analyze for microRNA and gene expression profiles. Compared with the sham ambulatory–no exercise group, 73, 79, and 80 microRNAs were altered 2-fold in the burn ambulatory, sham hindlimb unloading, and burn hindlimb unloading groups, all with no exercise, respectively. More than 70% of microRNAs were upregulated in response to burn and hindlimb unloading, whereas 60% microRNA of the profile decreased in hindlimb unloaded burn rats with exercise training. MiR-182 was the most affected in rat muscle. Gene ontology biological process and pathway analysis showed that the oxidative stress pathway was most stimulated in the hindlimb unloaded burn rats; while in response to exercise training, all genes in related pathways such as hypermetabolic, inflammation, and blood coagulation were alleviated. MicroRNAs and transcript gene profiles were altered in burn and hindlimb unloading groups, with additive effects on hindlimb unloaded burn rats. The altered genes' signal pathways were associated with muscle mass loss and function impairment. Muscle improvement with exercise training was observed in gene levels with microRNA alterations as well. (J Burn Care Res 2017;38:11–19)

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J.S., M.R.S., and A.R.C. contributed to data collection and analysis and manuscript preparation. L.A.B. was responsible for animal experiment, sample collection, and manuscript revision. C.E.W. and S.E.W. were integral to the concept and design of the experiment as well as the critical revision of the manuscript.

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Following a severe burn, patients suffer a hypercatabolic state in response to cytokine and stress hormone challenges. These stress signals, such as cortisol, catecholamine, and TNF-α, induce a catabolic/anabolic imbalance.[1] The body's flood of catabolic signals increases muscle breakdown and metabolism in muscle to call on its role as an energy reservoir. Patients with severe burn showed 83% muscle protein degradation and a 50% increase in protein synthesis. Alongside protein degradation, skeletal muscles demonstrate a net loss of amino acids, with a 50% increased transport into the blood and a 40% decreased transport from the blood.[2]

Scientists explored a robust biphase genomic profile in response to burn and sepsis from animals and patients.[3] In this multicenter study, they found that circulating leukocytes increase production of these inflammatory signals via a "genomic storm" of transcriptional changes. Genomic networks constructed

for several major pathways such as inflammation and proteolysis are precisely demonstrated in burn patients.

Epigenetic changes are caused by external environmental stimulations, which regulate the transcriptome but not the DNA sequence directly. Mechanisms of epigenetics include DNA methylation, histone modification microRNA (miRNA), etc. The miRNA is a conserved class of small (20–25 bases), abundant RNA-interfering (RNAi) molecules that inhibit gene expression at the translational level. It acts by transiently binding to the 3' UTR of messenger RNA (mRNA) with partial complementarity and by blocking their translation. At least 20 to 30% of protein-encoding genes in human are regulated by miRNAs, and these genes are often targeted by multiple miRNAs.[4] Clinically, miRNAs have been studied as markers for cancer progression, which are related to tumor cell growth or death through tumor-suppressor gene silencing. miRNAs are also involved in other disease states, such as type 2 diabetes, and posttraumatic stress disorder neural trauma injury.[5] The alteration of miRNA profiles were related to muscle disuse and atrophy,[6] and further affected with exercise.[7] Particularly in the area of burn, Liang et al[8] previously studied the miRNA profile of cells in burned dermis and found 66 miRNAs that were significantly up- or downregulated. However, there is little information regarding the mechanism of miRNAs mediating skeletal muscle atrophy after burn.

Due to the injury, burn patients are often in bed for extended periods of time.[9] Wu et al[10] demonstrated that rat muscle function decreased after burn and hindlimb unloading in an animal model. We speculated that miRNA changes in skeletal muscle after burn and bed rest contribute to muscle atrophy because of its abundance and sweeping range of actions on different genes.

Exercise studies have shown to positively mitigate muscle atrophy.[11] In pediatric burn patients, beneficial improvement of muscle mass was achieved with a combination of aerobic and resistance exercise training.[12,13] We recently studied the effects of resistance exercise on muscle function recovery in burn rats with hindlimb unloading.[14] There was the question of whether gene expression alteration with exercise training is correlated with miRNA regulation. Therefore, we further hypothesized that exercise improves muscle pathophysiological change that is associated with epigenetics-regulated gene expression after burn and muscle disuse. The purpose of the current study was to characterize the miRNA and genomic profile in burn and hindlimb unloaded animals with exercise training.

METHODS

Forty-eight adult male Sprague-Dawley rats (Envigo [Harlan Labs], Indianapolis, IN) were used in this study. The animals' protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Houston in accordance with NIH guidelines. All animal procedures were performed at University of Texas Health Science Center at Houston and fully described previously.[14] The experiment flow chart is presented in Figure 1 and briefly addressed below.

Burn Procedure

All animals received a full-thickness scald burn of 40% TBSA under 2 to 4% isoflurane anesthesia. The burned animals (B) were resuscitated with 20 ml of intraperitoneal lactated Ringer's with buprenorphine for analgesia treatment. Sham animals (S) received the same procedure except for the scald burn.

Hindlimb Unloading

Animals were placed in a hindlimb unloading system described by Morey-Holton and Globus after burn or sham injury.[15] Rats were able to freely access regular chow (Harlan Teklad #2018) and water without the hindlimbs contacting the walls of the cage. Animals in the ambulatory groups (A) were housed in similar cages but without hindlimb unloading.

Resistance Exercise

On the day of injury, animals were trained (E) to climb 1 m at an 80° incline with tail weights five times twice daily. Weights were calculated as percent body mass of each individual rat and gradually increased in increments of 10% every few days as tolerated with a maximum weight of 50% body mass. All animals including the nonexercised group (N) were pretrained 10 days before injury.

Total RNA Extraction and Genomic Analysis

On day 14 after injury, all animals were killed, and the hindlimb muscles on the right were harvested and weighed. Half the plantaris tissue was immersed in RNA stabilization reagent (Qiagen, Hilden, Germany) and stored at −80°C. Tissue samples from three animals in each group were pooled, and total RNA was extracted using Qiagen miRNeasy Mini Kit. RNA purity was more than 99%, and 1 μg of RNA sample was processed at the UT Southwestern Microarray Core facility for the following miRNA and gene expression measurements.

The Affymetrix miRNA 4.0 Arrays chip (Santa Clara, CA) was applied for miRNA detection. The chips' reproducibility (intralot and interlot) is greater than 0.95. A total of 36,333 small noncoding RNA

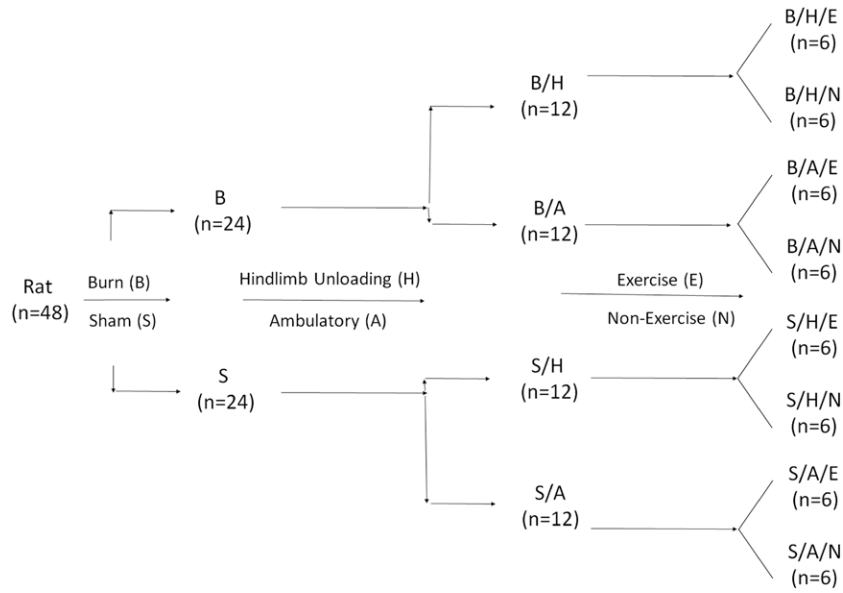

Figure 1. Flow chart of animal experiment that outlines the size of animal and the order of operation.

probes, including varied species and controls are coated in one chip for each sample. Rat gene expression from each pooled sample was detected in triplicate using the Affymetrix Rat Gene 2.0 chip. The chip contains 30,429 rat gene probes. Raw signal intensity data were normalized with robust multi-array average from the Affymetrix data bank. The raw intensity values ratio of signal intensity were background corrected, log 2 transformed, and then quantile normalized. A linear model was then fitted to the normalized data to obtain an expression measure for each probe set on each array. The linear fold change of signal data was analyzed with Affymetrix Transcriptome Analysis Console 3.0 software. Threshold filters were set as the default value for both miRNA and gene expression data analysis. The absolute linear fold change value was greater than two. For triplicated genomic sample data analysis, a one-way between-subject analysis of variance (unpaired) was further applied with significant acceptance of $P < .05$. The interaction of miRNA and target genes, gene ontology (GO) biological processing, and related pathways were also analyzed.

RESULTS

General Description of miRNA and Gene Expressions

There were 1218 rat species miRNAs in a total of 36,333 miRNAs detected in rat muscle samples, including 728 rat mature miRNA probe sets and 490 pre-miRNA probes. Most signal intensities were lower than two in all groups. The highest probe signal intensity (binary log ratio) was 15.4 for miRNA-206-3p in all groups. We observed 74.5% of transcripts in the S/A/N group and 73.9% of the B/A/N group with a signal intensity of less than two. There were 73, 79, and 80 miRNAs altered in the B/A/N, S/H/N, and B/H/N groups, respectively, compared with the S/A/N group. More than 70% of the miRNAs were upregulated in response to burn and hindlimb unloading, whereas about 60% of miRNAs were upregulated in B/H rats with exercise training (Figure 2A).

There were 30,429 rat genes detected using the Rat Gene 2.0 chip. GAPDH (transcript cluster ID 17799923) demonstrated the greatest signal intensity (13.5 binary log ratio). Filtered by default threshold values, there were 47 and 135 genes altered in rat muscle with burn or hindlimb unloading, respectively, whereas 239 genes were disturbed in combined burn and hindlimb unloading rats. There were 2, 20, and 22 genes that increased greater than 4-fold in the B/A, S/H, and B/H groups separately. In contrast, 62 of 71 genes decreased in the B/H/E vs B/H/N group (Figure 2B).

MiRNA and gene expression profiles are distinguished in response to burn, hindlimb unloading, and exercise and described separately below.

The miRNA and Gene Expression Profile in Response to Burn (B/A/N vs S/A/N)

In all, 79.4% of 73 miRNAs were upregulated in burn animals. The amplitude of upregulated

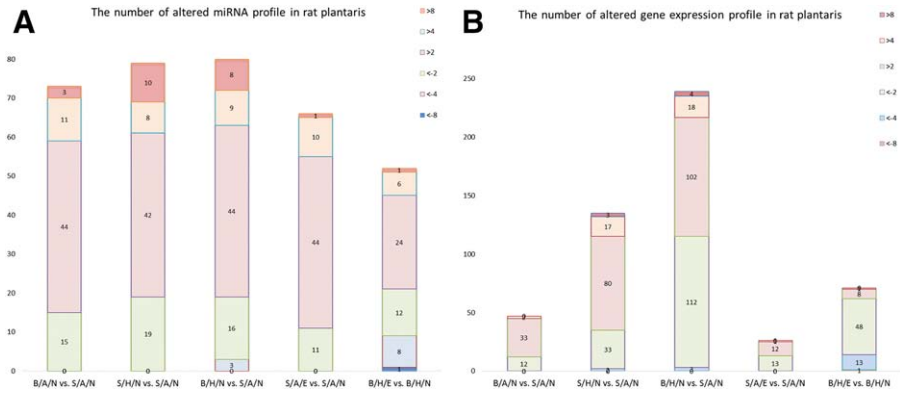

Figure 2. Stacked bar figures showing the number of (A) miRNA and (B) genes in rat plantaris altered in response to burn (B/A/N vs S/A/N), hindlimb unloading (S/H/N vs S/A/N), combination of burn and hindlimb unloading (B/H/N vs S/A/N), exercise in normal rats (S/A/E vs S/A/N), and exercise in burn and hindlimb unloaded rats (B/H/E vs B/H/N).

miRNAs was higher than that of downregulated ones. There were 14 miRNAs upregulated more than 4-fold, including the three most upregulated miRNAs, miR-182 (12.81), miR-184 (10.50), and miR-155-5p (8.82). Fold changes were less than three in all 15 downregulated miRNAs. The miR-409a-3p was the most decreased (–2.95) in burn animals (see Supplementary Table 1-1-burn, Supplemental Digital Content, at http://links.lww.com/BCR/A71).

There were 47 genes changed, 12 down- and 35 upregulated, in the B/A/N group compared with the S/A/N group (see Supplementary Table 1-2-burn, Supplemental Digital Content, at http://links.lww.com/BCR/A72). Thirty-two changed genes were associated with multiple GO biological processes, and six signal pathways were altered. The ketone body metabolism, inflammatory response, and striated muscle contraction pathways were most activated (see Supplementary Table 1-3-burn, Supplemental Digital Content, at http://links.lww.com/BCR/A73).

In viewing the interaction network, the most upregulated miRNA, miR-182, decreased the *col1a2* gene, which collaborates with downregulated miR-409-3p. MiR-182 also works with miR-193-3p and 125-b-1p to downregulate the *neu2* gene, which participates in muscle cell differentiation. The second most upregulated miRNA, miR-184, was associated with the decreased *obp3* and *chad* genes (which are associated with GO biological processes of small molecular transportation and cartilage condensation, respectively), and also collaborates with other miRNAs (Figure 3).

The miRNA and Gene Expression Profile in Rats With Hindlimb Unloading (S/H/N vs S/A/N)

There were 79 total altered miRNAs, including 19 downregulated and 60 upregulated ones. The amplitude of upregulated miRNAs was greater than that of the downregulated. Eighteen of the miRNAs' linear fold changes were upregulated more than 4-fold, and 10 miRNAs even changed more than 8-fold; in contrast, downregulated miRNAs were all changed less than 4-fold. MiR-182 (23.83), miR-184 (17.73), miR-183-5p (16.61), and miR-122-5p (14.19) were the most upregulated in the S/H/N group, and the most downregulated miRNAs included miR-489-3p (-3.54), miR-665 (-3.21), and miR-675-5p (-3.19) (see Supplementary Table 2-1-HLU, Supplemental Digital Content, at http://links.lww.com/BCR/A74).

There were 135 genes, 100 up- and 35 downregulated, in rat muscle in response to hindlimb unloading (see Supplementary Table 2-2-HLU, Supplemental Digital Content, at http://links.lww.com/BCR/A75). In all, 20 genes increased more than 4-fold; only two genes decreased more than 4-fold. Seventy-nine genes had varied biological process functions involving nine pathways, with the three most prominent pathways being the mitogen-activated protein kinase (MAPK) cascade, the blood-clotting cascade, and fatty acid synthesis (see Supplementary Table 2-3-HLU, Supplemental Digital Content, at http://links.lww.com/BCR/A76).

In viewing the interaction network, a large complex network was constructed between miRNAs

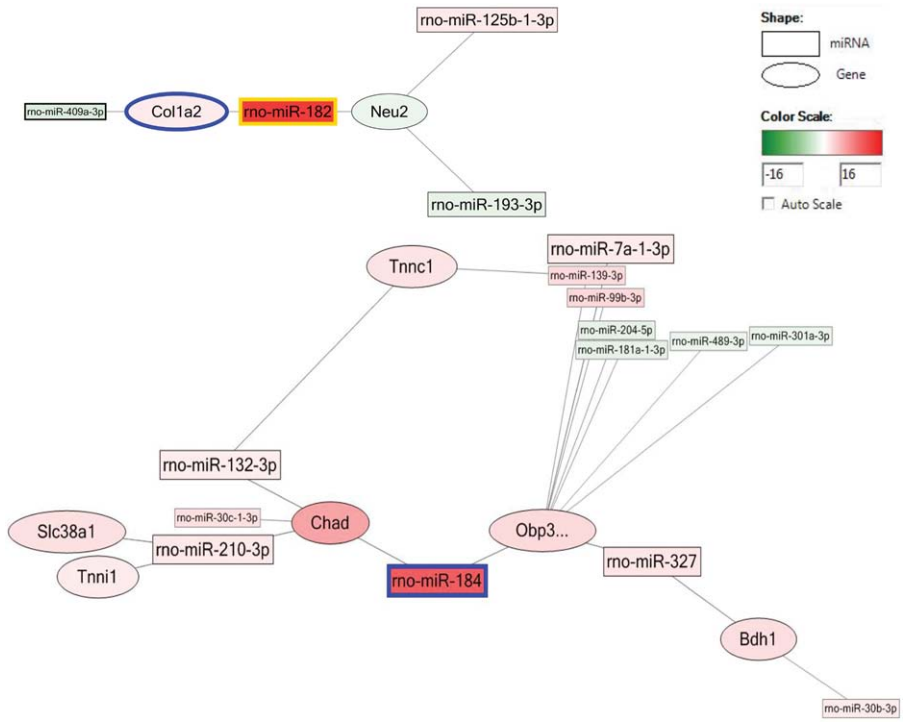

Figure 3. The network of miRNA and related genes after burn (B/A/N vs S/A/N). Square shape stands for miRNA, and oval shape stands for gene. Pseudocolor from green to red stands for fold change from –16 to 16.

and genes demonstrating altered expression. The most increased miRNA, miR-182, upregulated the *clnd19* gene (for neuronal action potential propagation) in collaboration with decreased miR-489-3p. MiR-182 also worked with other miRNAs such as miR-335 and miR-484 to decrease the *neu2* gene (for myotube differentiation positive regulation). The second most upregulated miRNA, miR-184, worked with miR-342 and miR-484 to increase *obp3* and decrease *cib2* and *plcd4* genes, which are both for calcium ion binding (Figure 4).

The miRNA and Gene Expression Profile in Rats With Combined Burn and Hindlimb Unloading (B/H/N vs S/A/N)

We found 80 miRNAs, including 61 upregulated miRNAs and 19 downregulated miRNAs, with only three downregulated more than 4-fold. There were 17 miRNAs upregulated more than 4-fold, and miR-182 even increased 35-fold (see Supplementary Table 3-1-bh, Supplemental Digital Content, at http://links.lww.com/BCR/A77).

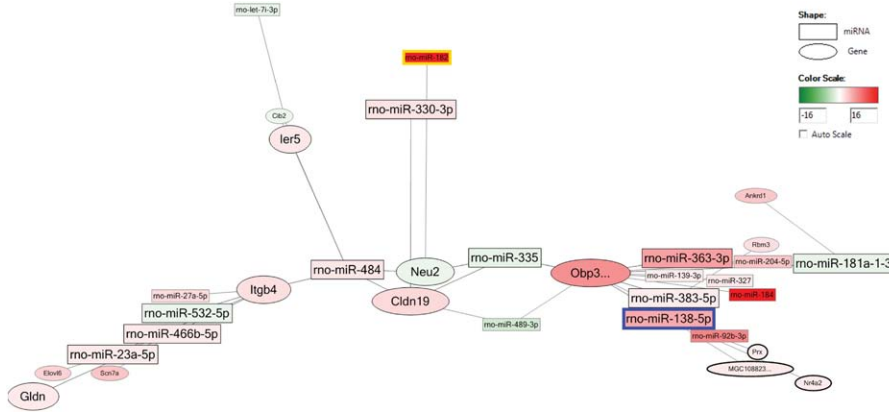

Figure 4. The network of miRNA and related genes after hindlimb unloading (S/H/N vs S/A/N). Square shape stands for miRNA, and oval shape stands for gene. Pseudocolor from green to red stands for fold change from –16 to 16.

There were 239 genes altered between the B/H/N vs S/A/N group. Only three of 115 downregulated genes changed more than 4-fold, whereas there were 22 of 124 genes upregulated more than 4-fold (see Supplementary Table 3-2-bh, Supplemental Digital Content, at http://links.lww.com/BCR/A78). Overall, 165 genes related to multiple GO biological processes were involved in 14 pathways. Oxidative stress, the MAPK cascade, fatty acid synthesis, and the blood-clotting pathway were activated, and cardiovascular signaling and the p53 signal pathway were inhibited (see Supplementary Table 3-3-bh, Supplemental Digital Content, at http://links.lww.com/BCR/A79).

In viewing the interaction network, there were independent and additive effects in miRNA and gene expression levels when comparing the effects of burn and hindlimb unloading factors. While miR-409a-3p decreased 2.95-fold in B/A/N without detection in S/H/N mice, in B/H/N, miR-409a-5p decreased 2.03-fold. MiR-92b-3p only demonstrated an 8.04-fold upregulation in the S/H/N group but increased to a 9.46-fold upregulation in the B/H/N group. Most strikingly, miR-182 has an additive increased effect with combined burn and hindlimb unloading (35.35) compared with burn (12.81) and hindlimb unloading (23.82) individually. At the transcriptional level, *Fmod* only increased in burn rats but not in rats with hindlimb unloading and the *Mbp* gene increased in an opposite manner, while Nr4a3 had an additive effect in response to the combination of burn and hindlimb unloading (see Supplementary Table 4-bh effect, Supplemental Digital Content, at http://links.lww.com/BCR/A80).

The miRNA and Gene Expression Profile in Rats With Burn and Hindlimb Unloading in Response to Exercise Training (B/H/E vs B/H/N)

There were 52 miRNAs altered, including 21 down- and 31 upregulated, in burn and hindlimb unloading rats with exercise training. There were nine miRNAs downregulated more than 4-fold, whereas seven were upregulated more than 4-fold. The most upregulated miRNAs include miR-1843-3p (8.52), miR-495 (5.92), and miR-6324 (5.78) (see Supplementary Table 5-1-bhE, Supplemental Digital Content, at http://links.lww.com/BCR/A81).

In viewing the interaction network, there were 71 gene changes, with 62 downregulated genes and only nine genes upregulated less than 4-fold in transcriptional level (see Supplementary Table 5-2-bhE, Supplemental Digital Content, at http://links.lww.com/BCR/A82). Overall, 54 genes partook in different biological processes and seven pathways. The MAPK cascade, fatty acid synthesis, and the inflammation response pathway were the top three alleviated pathways (see Supplementary Table 5-3-bhE, Supplemental Digital Content, at http://links.lww.com/BCR/A83).

We further estimated the different effect of exercise between burn rats with hindlimb unloading and sham controlled rats. In S/A/E rats, there were 66 miRNAs altered, with 11 downregulated and 55 upregulated. We found that miR-182 increased 10.06-fold in S/A/E rats. There were only 12 genes altered, with seven upregulated and five downregulated. Although nine of these genes had functions in various biological processes, no pathway change was observed in normal rats with exercise training.

DISCUSSION

In this study, we characterized the miRNA and gene profiles in rat plantaris under conditions of burn, hindlimb unloading, and resistance exercise. The complexity of this regulation's network is displayed at both the epigenetic and transcriptome levels. Those consequent changes of biological processing and involved signal pathways reflect muscle pathophysiological changes in response to burn and hindlimb unloading. Hypermetabolic response ketone bodies KEGG and glucose, and inflammation response were activated after burn injury; oxidative stress, MAPK cascade, and fat acid synthesis pathways were stimulated by hindlimb unloading; and striated muscle contraction and blood clotting pathways were stimulated by both burn and hindlimb unloading. Resistance exercise altered the transcriptome profile associated with muscle structure and function improvement (Figure 5).

Cells respond to the body's stress signals by making coordinated changes in gene expression. Padfield et al[16] reported the genomic profile in mice mouse muscle by 3 days after burn, including muscle development and function, inflammation and acute-phase immune response, amino acid and protein synthesis, and energy metabolism pathways. Vemula et al[17] verified this, linking 28% of the changed genes to metabolism. These included genes responsible for triglyceride utilization, fatty acid import, and acute-phase proteins. Merritt et al[18] reported that the inflammatory response activated stat/NFκβ to calcium-mediated proteolysis and ubiquitin–proteasome with absence of protein synthesis inhibition. In addition, we found that muscle mass loss is

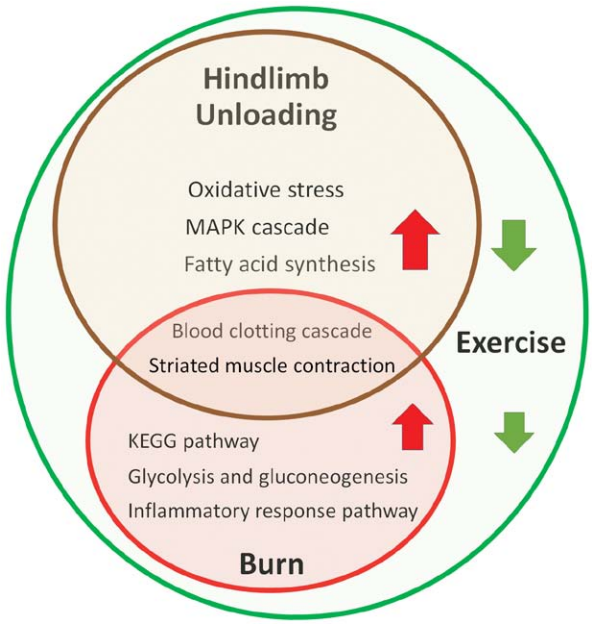

Figure 5. The scheme of affected signal pathways in response to burn, hindlimb unloading, and exercise training. Red arrows indicated activated pathways. Green arrows indicate inhibited pathways.

associated with insufficient myogenesis in response to burn and that TNF-α as a pro-inflammatory cytokine plays an important role in inhibiting muscle myogenesis.[19] Although samples were collected 14 days after burn in the current study, we still observed increased numbers and amplitudes of gene and miRNA profile changes. Furthermore, we found those affected genes were mainly related to the metabolic and inflammatory response signal pathways. We are therefore not surprised by Jeschke et al's[20] previous report that the hypermetabolic status can even last for years in burn patients.

In human patients with bed rest, a gene profile reveals changes in energy pathways: oxidative phosphorylation, tricarboxylic acid cycle, organic compound usage, and carbohydrate metabolism.[21] Bonaldo and Sandri[22] revealed the intracellular mechanism by which hindlimb unloading activates cell apoptosis in an NF-KB-dependent manner. In the current study, we found that burn mainly affects inflammation and metabolic pathways in rats, such as ketone bodies synthesis and degradation, inflammatory response, striated muscle contraction, and glucose metabolism; hindlimb unloading affects muscle signal pathways, including oxidative stress, MAPK cascade, fatty acid synthesis, and blood clotting cascade pathways; and *Mal*, *Hmox1*, and *Btg2* genes are directly related to GO biological process of cell apoptosis. Severe burn and disuse have independent roles in body composition change.[23] Therefore, it is logically believed that burn and hindlimb unloading might activate different major pathways with distinguishable characteristic profiles.

Muscle disuse amplified muscle function impairment in severely burned rats. In plantaris, the tissue's wet weight significantly decreased in response to burn and hindlimb unloading, respectively. Normalized to body mass, the tissue weight still decreased between the ambulatory and hindlimb unloading with decreased twitch and tetanic forces.[10] In our current study, we observed that muscle disuse affects more genes in rat muscle than burn, so hindlimb unloading could be more closely associated with muscle impairment and function loss than burn. Furthermore, an overlap of miRNA and gene changes were observed in burn rats with hindlimb unloading. The double factors of burn and hindlimb unloading could amplify the signal strength and extend the pathophysiological phenotype in muscle.

The current study is the first to investigate miRNA profiles related to their target genes in muscle atrophy after burn. The miRNA differs from a similar class of RNAi, short interfering RNA (siRNA), in that it does not usually cleave the complement mRNA or affect gene transcription. The importance of miRNAs has been observed in regulating skeletal myogenesis. MiRNAs are highly enriched in skeletal muscle and participate in skeletal myogenesis and muscle regeneration.[24] In addition, miRNA-1 improves myogenic differentiation by inhibiting histone deacetylate 4, and miR-133a increases myoblast proliferation by repressing serum response factor.[25] Cardiomyocyte hypertrophy can be induced by miR-195, and miR-195 with other 4 miRNAs increased in human heart failure and upregulated during cardiac hypertrophy in vivo.[26]

Exercise affects gene expression through miRNA changes. Following 90 minutes of exhaustive endurance exercise (forced treadmill running) in mice, miR-1 and miR-181, both thought to increase muscle differentiation and development, and miR-107 were increased.[7] Resistance exercise training reduced anabolic signaling with gene alteration, including hypertrophic growth, protein degradation, and angiogenesis.[27] In another clinical study, investigators distinguished an miRNA profile from human vastus lateralis with a 5 day/week resistance exercise for 12 weeks, and speculated those miRNAs served as compensatory mechanisms.[28] We observed that there was a complicated network of epigenetic regulation in the current study. Not just one but several miRNAs control one single gene, and a single miRNA is also involved in multiple genes' regulation. It is therefore better to examine the whole profiles of miRNA and

the related genes, and furthermore understand the protein structure and function change.

Previous studies showed that miR-182 has multiple functions as a regulator of apoptosis, growth, and differentiation programs. Kouri et al[29] reported that the injection of synthesized miR-182-based spherical nucleic acids suppressed tumor glioma burden and increased animal survival. More interestingly, miR-182 was shown to prevent skeletal muscle atrophy by interfering with forkhead box O3 (FoxO3) mRNA. The miR-182 decreased FoxO3 expression in C2C12 with further inhibition of atrohgin-1 and ATG12.[30] Overall, miR-182 was the most phenomenally affected miRNA within all treatment groups in the current study. It increased 12.8- and 23.8-fold in burn and hindlimb unloading, respectively, and additively 35.5-fold in B/H/N rats. Exercise training decreased its expression 7.8-fold afterwards.

Burn causes a hypermetabolic status with a hyperinflammation response, and muscle is a key participant in the systemic metabolic response. The current study could provide novel insight into potential target treatment at the epigenetic level. For instance, we have shown insulin resistance in animal models and burn patients.[31] One review paper discussed the possibility of targeting miRNA to treat insulin resistance in burn patients.[32]

In summary, miRNAs and transcript gene profiles in rat plantaris were affected in burn and hindlimb unloading. These changes seen in signal pathways are associated with muscle pathophysiological changes, including muscle mass loss and function impairment. The muscle improvement observed with exercise training was also observed at the gene level with miRNA and genomic pathway alterations. The current exploration of regulation networks involving epigenetics and gene pathophysiological changes might aide the development of future biomarkers and potential therapeutic development in patients with muscle atrophy.

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OPEN

SEVERE BURN-INDUCED INFLAMMATION AND REMODELING OF ACHILLES TENDON IN A RAT MODEL

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ABSTRACT—Severe burn causes systemic inflammation and hypercatabolism, resulting in damage to multiple organs distant to the burn site, including the musculoskeletal system. Bone mass and muscle loss have been reported. However, tendon that connects bone and muscle has not been studied in comparable detail. Here we aimed to characterize the molecular and functional changes in Achilles tendon triggered by severe burn. Forty male Sprague–Dawley rats received 40% total body surface area scald burn. Achilles tendons were collected up to 14 days postburn. Sham-treated animals served as a control group. We analyzed tendons for changes in expression of IL-6, IL-1β, TNF, MMP9, MMP13, TGFβ1, Collagens I and III, and for morphological and biomechanical changes. Gene expression of IL-6 and IL-1β as well as MMP9 and MMP13 increased in rat tendon 3 days after burn. Col3a1 increased at day 3 and col1a1 at day 7. At day 14, TGFβ1 increased, whereas the protein ratio for collagens I/III decreased, indicating tendon remodeling. Histological analysis with H&E and Picrosirius red staining further revealed a decrease in organized collagen fibers 14 days after burn. Biomechanical analysis showed a decrease in stiffness and ultimate force of tendons in burn rats. We conclude that tendinopathy was observed in Achilles tendon 14 days after severe burn, via the induction of inflammation and remodeling. The present study provides a model of tendinopathy that may be used for the development of therapeutic approaches after burn.

KEYWORDS—Biomechanical property, collagen alignment, tendinopathy, thermal injury

INTRODUCTION

Severe burn is defined as a full-thickness burn of greater than 30% of total body surface area (TBSA). It occurs at a rate of approximately 5/100,000 persons per year globally and may affect multiple organs, even those distantly located from the burn site (1). In the musculoskeletal system, severe burn induces hypercatabolism in muscle and bone due to the activation of systemic inflammation, and disuse from long immobilization periods (2–6). Muscle loss and atrophy postburn have been associated with increased levels of TNF-α (7), whereas bone mass loss has been associated with circulating TNF-α, IL-1β, and IL-6, which are increased shortly after burn (8–10). Despite the findings of mass loss in both skeletal muscle and bone, there are limited studies on the molecular and structural effects of burn injury on tendon.

Tendon injuries are common events occurring in sports and other activities (11–13). Tendons are mostly composed of type I collagen fibrils aggregated together into fibers to provide tensile strength (14). There are also type III collagen fibrils intercalated into the collagen I fibers (14). When tendon experiences remodeling or injury, collagen III content increases, resulting in a decrease in tensile strength (15). On the contrary, metalloproteinases such as the collagenase MMP13 can cleave the collagen triple helix, creating fragments that are further degraded by gelatinases such as MMP9 (16). In addition, modulators of musculoskeletal growth and differentiation, such as TGFβ1, have been reported to coincide with scar formation and healing in tendon (17).

In this study, we hypothesize that the systemic inflammation caused by severe burn will induce molecular and structural changes in Achilles tendon that ultimately debilitate this tissue. By understanding the local changes produced in tendon, it is possible to adopt additional preventive measures to improve healing and to aid with physical therapies that target muscle and bone.

MATERIALS AND METHODS

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas in accordance with the Institutional and Association for Assessment and Accreditation of Laboratory Animal Care guidelines and in accordance with NIH guidelines. All animal procedures were carried out at the UT Southwestern

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Authors' contributions: PH, JS, and SW designed the experiments and project. PH, DB, TM, JW, RH, DC, and JS performed the experiments. PH, HL, JW, KM, TW, and JS analyzed the data. All authors were involved in drafting and revising the manuscript.

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animal facility. A total of 40 adult male Sprague–Dawley rats (Charles Rivers), 270–300 g, were acclimated in an animal facility a week before the experiment. Animals were fed a pellet diet (Harlan Teklad #2018) *ad libitum* and housed in a reversed 12-h light/dark cycle with maintained room temperature.

Burn procedure

Animals were randomly divided into five groups: control (n = 11), and 1 day (n = 6), 3 days (n = 6), 7 days (n = 6), and 14 days (n = 11) postburn. Under 2% to 4% isoflurane inhalation anesthesia, animal hair was removed from the dorsal and lateral surfaces and the animals were secured in a specifically constructed template device with opening size of 10 cm length and 7 cm wide (curved). Dorsal skin amounting to 40% of TBSA was exposed through the device aperture and immersed in 95°C to 100°C water for 10 s. The burned animals then received intraperitoneally 4 mL/kg body weight/TBSA% of lactated Ringer's solution for resuscitation immediately after injury. Animals were given analgesia intraperitoneally (buprenorphine HCl 0.05 mg/kg) immediately and 12 h postburn. Control animals received sham treatment with the procedure of hair removal, anesthesia, and submersion in room temperature water. Control animals were not subjected to resuscitation procedure because injecting this amount of liquid intraperitoneally will cause cardiac failure and/or abdominal compartmentalization syndrome that would be fatal.

Tissue harvesting

Animals were euthanized with anesthetic overdose. Achilles tendons were isolated from surrounding tissue and dissected from both sides of hind limbs. The proximal ends of the Achilles tendons were disconnected at the end of the gastrocnemius, plantaris, and soleus, and the distal ends at the calcaneus. Left leg tendons of eight control rats and six burn rats for each time point (1, 3, 7, and 14 days) were stored in RNAlater (Qiagen), whereas right leg tendons were snap frozen for protein extraction. Samples were stored in −80°C for further analysis. Tendon RNA was obtained with RNAeasy Universal kit (Qiagen). cDNA conversion was done with iScript kit (Bio-Rad), and qPCR with SsoFast Eva Green kit (Bio-Rad). Primers for IL-6, TNF, IL-1β, col1a1, col3a1, MMP9, MMP13, and TGFβ1 were purchased from QuantiTect Primers (Qiagen). Gene expression was calculated using the ΔΔCt method with 18 s as control.

For protein extraction, tendon tissue was homogenized in RIPA buffer (Invitrogen) with proteinase inhibitor cocktail (Sigma). Protein quantification was performed with BCA assay (Pierce); 10 μg of total protein was used for Western blot. Antibodies included anti-collagen I (Abcam), anti-collagen III (Abcam), goat anti-mouse HRP (Pierce), and goat anti-rabbit HRP (Pierce), which were prepared in 2% BSA. Blots were developed with ECL (Pierce) using a CCD camera system.

Histology

Right leg Achilles tendons were dissected from three control rats and five burn rats (14 days). Parts of the calcaneus bone and gastrocnemius muscle were included. Tendons were fixed in 10% neutralized buffer formalin and decalcified with 10% formic acid. Paraffin sections of 5 μm were processed for hematoxylin and eosin (H&E) staining, as well as Picrosirius red staining. Samples were visualized in a Nikon Eclipse Ti microscope or in an Olympus BH2-RFCA using polarized light. The image analysis for Picrosirius red was performed using ImageJ (National Institutes of Health (18)). Several regions of 320 × 320 pixels were selected from the central area of each tendon. After converting the image into 8-bit and 256 color, the measurements of red, green, and blue colors were postprocessed in Excel. For each piece, the (green + blue)/red values were calculated and grouped as control vs. 14 days postburn, where green + blue represent more organized fibers compared with red.

Biomechanics

Three tendons from the control group and five tendons from the burn rats at 14 days postburn were dissected, leaving part of muscle and foot, and wrapped with cold PBS-soaked gauze to prevent dehydration. Sandpaper was fixed to both tendon extremities. Specimens were then clamped and tested using an Instron 5565 universal testing system (Instron Corp., Norwood, MA) equipped with a 5-kN load cell. After precondition using a cyclic load oscillating from 0% to 2.5% strain for 20 cycles, samples were finally pulled until failure at a crosshead speed of 6 mm/min collecting force and deformation data throughout the test. Tendon material properties, ultimate tensile force, and stiffness were calculated from the force-deformation curve.

Statistics

Data are presented as mean ± error propagation for gene expression and mean ± standard deviation elsewhere. Data were analyzed in GraphPad Prism 7 with one-way ANOVA and Fisher LSD posthoc test, or by unpaired Student *t* test when comparing two variables ($P < 0.05$ being significant).

RESULTS

Changes in gene expression of cytokines, MMPs, and TGFβ1 in Achilles tendon after burn

The gene expression of the proinflammatory cytokines in tendon tissue IL-1β and IL-6 was affected by severe burn. Both increased and reached a peak level at 3 days postburn, being significantly higher compared with control group, $P < 0.05$. The expression of TNF was also higher than control but did not reach a significant level. After 7 days, all three genes decreased back to control levels (Fig. 1A).

We next analyzed whether the expression of the matrix metalloproteinases commonly affected in tendinopathy were altered by burn. The results showed that the expression of both MMP9 and MMP13 continually increased after the injury and reached significantly higher levels at 3 days postburn compared with the control group, $pP < 0.05$ (Fig. 1B). MMP9 showed a second significant increase at 14 days postburn by more than 20-fold compared with control, $P < 0.05$ (Fig. 1B).

TGFβ1, which has been linked to tendon repair and healing, was also analyzed. We found a significant upregulation at day 14 postburn ($P < 0.05$) (Fig. 1C).

Changes in collagen expression and collagen I/III ratio

The gene expression of col3a1 significantly increased at day 3 postburn, whereas col1a1 did it at day 7 postburn (Fig. 1D). Protein levels of collagen I were significantly lower at day 7 postburn, $P < 0.05$ (Fig. 1E) and were restored back to control levels by day 14. Collagen III protein, on the contrary, increased significantly at day 14 postburn, $P < 0.05$ (Fig. 1E). The protein ratio of collagens I/III showed a significant decrease at day 14 postburn (Fig. 1F).

Histological and biomechanical evidence of tendon remodeling

We analyzed the control and 14-day postburn tendons stained with H&E to further investigate signs of remodeling. Tenocytes and extracellular matrix fibers were found aligned with the longitudinal axis in the control group. Most of the nuclei of tenocytes showed an elongated shape. In contrast, tendons from animals at 14 days postburn showed less parallel organization of cells and fibers, as well as hypercellularity with cell aggregation and round nuclei (Fig. 2A). To observe collagen organization in more detail, tendon sections were stained with Picrosirius red and analyzed under polarized light. A representative image of a control rat showed that collagen fibers were more organized and well aligned compared with 14 days postburn, where tendon fibers showed less organization (Fig. 2B). Our method of quantification of green, blue, and red colors showed that the (green + blue)/red ratio significantly decreased 14 days postburn, $P < 0.05$ (Fig. 2C).

To assess tendon function in parallel with histological changes, we measured the ultimate force of the left tendons of the same control and 14-day postburn rats utilized for histology (Fig. 3A). Data obtained from three control rats and five burn rats showed that control tendons had a higher

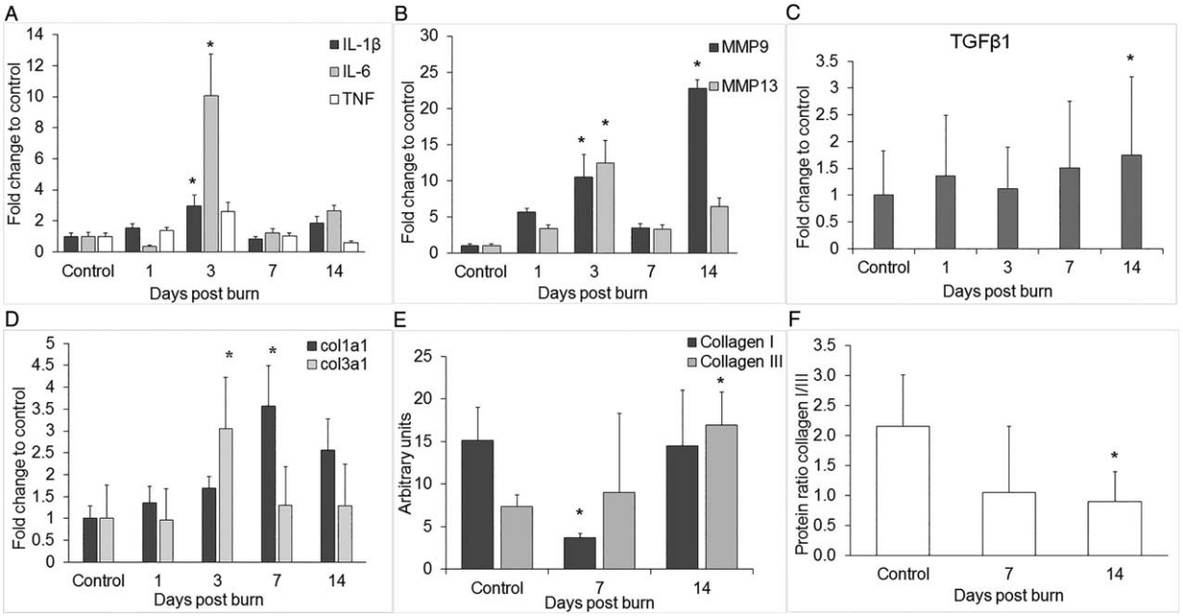


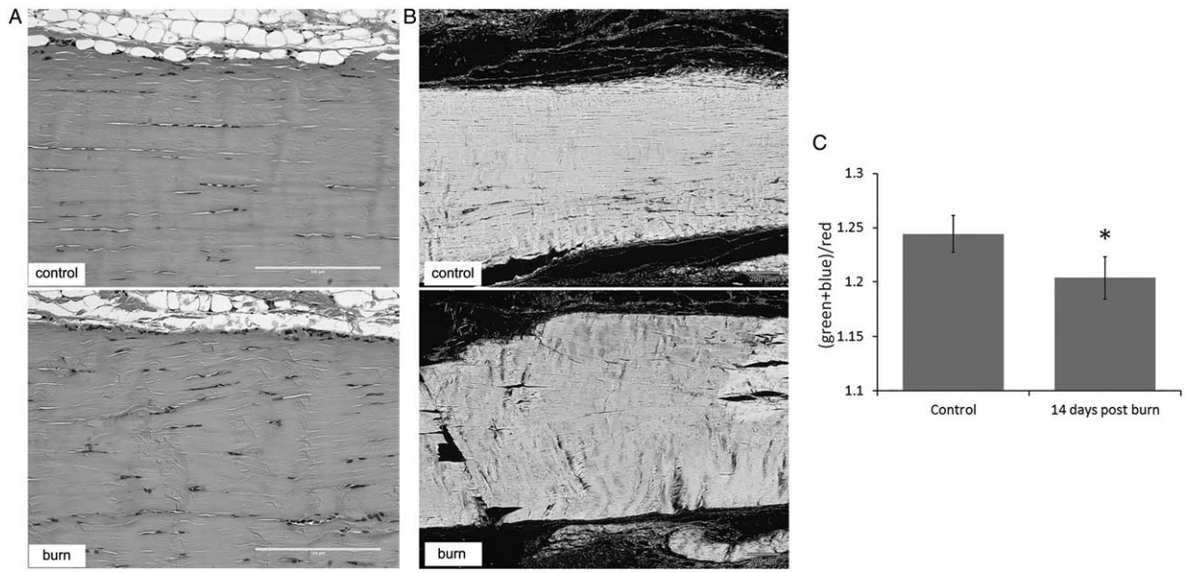
Fig. 1. **Changes in expression of proinflammatory cytokines, metalloproteases, and collagens in Achilles tendon after severe burn.** (A) Gene expression of cytokines IL-1β, IL-6, and TNF (B) metalloproteinases MMP9 and MMP13, (C) TGFβ1 and (D) collagens col1a1 and col3a1 were examined with qPCR. (E) Protein levels of collagen I and collagen III obtained by Western blot and normalized to total protein (F). Collagen I/III protein ratio was calculated from the Western blot results. Data are shown as mean with error propagation in (A) and (B). Data are shown as mean ± SD in (C) and (D). $^{*}P < 0.05$ vs. control.

ultimate tensile force or maximum force before failure (Fig. 3B). The average ultimate force for the control group was 43.7 ± 3.0 N, whereas tendons of the 14-day postburn group showed an average ultimate force of less than half that of the controls at 19.9 ± 9.7 N, $P < 0.05$.

We also calculated tendon stiffness, which is the amount of measured deformation from an applied force. Control tendons had an average stiffness of 19.38 ± 0.16 N/mm that is significantly different from 14-day postburn rats with an average stiffness of 9.18 ± 0.99 N/mm, $P < 0.001$ (Fig. 3C).

DISCUSSION

In accordance with an induction of acute inflammation, we observed a significant increase in gene expression of the proinflammatory cytokines IL-1β and IL-6 as well as the



AQ4 Fig. 2. **Histological evidence of changes in Achilles tendon after severe burn.** (A) Tendons from control rats (upper panel) and 14-day postburn rats (lower panel) were stained with hematoxylin and eosin to show cell morphology and gross fiber organization. (B) Slides from the same tendons were used for Picrosirius red staining and analyzed with polarized light to show fiber organization of control (upper panel) vs. 14-day postburn rats (lower panel). (C) Quantification of (green + blue)/red of the Picrosirius red images under polarized light was used to determine organization of fibers. Scale bars = 200 μm. $^{*}P < 0.05$ vs. control.

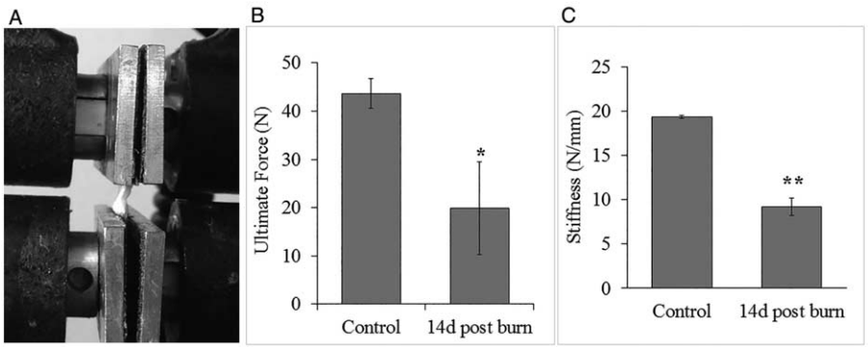


Fig. 3. **Biomechanical changes in Achilles tendon after severe burn.** (A) Example of tendon set-up for biomechanical testing. Tendons are pulled until failure. (B) Maximum load values obtained from the force-deformation curve of three control rats and five injured rats 14 days postburn. (C) Stiffness values calculated from force-deformation curves comparing no-burn vs. 14-day postburn tendons. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control.

matrix metalloproteinases involved in remodeling, MMP9 and MMP13. We observed a decrease in collagen I/III protein expression, upregulation of TGFβ1, histological signs of remodeling, and a decrease in ultimate tensile strength of tendon 14 days postburn. Taken together, these results demonstrate, for the first time, the presence of inflammation and remodeling in tendon distant from the burn site after severe burn. This is consistent with similar findings in the literature for both skeletal muscle and bone (1–4, 7).

Serum levels of IL6 have been previously reported to increase rapidly after burn and stay higher than nonburn for several days (10, 19). The local increase in IL-1β and IL-6 expression that we observed in tendons indicates an inflammatory response in this tissue. IL-6 has been suggested to also act as a growth factor by stimulating the synthesis of collagen in response to mechanical loading (20). Therefore, the rise of both IL-1β and IL-6 levels points toward the initiation of acute inflammation in the tendon with a concomitant induction of collagen synthesis.

Previous reports have shown the involvement of IL-1β in the upregulation of MMPs and tissue remodeling in tendon (21, 22). Even though MMPs are necessary for normal tissue homeostasis, an imbalance in the activity of these metalloproteases has been shown to be detrimental to tissue healing after injury, as an improvement of Achilles tendon repair can be obtained by the use of the nonselective MMP inhibitor, doxycycline (23). More investigation is needed to assess whether the increase in proinflammatory cytokines is mediated by tenocytes or by macrophage infiltration into the tissue after generation of the systemic response and to evaluate chronic inflammation at later time points.

Our results revealed a significant decrease in the collagen I protein level at 7 days postburn, with a subsequent increase akin to the control level by day 14. We propose that the initial decrease in collagen I can be explained by the upregulation of MMP13 and MMP9, generated by IL-1β. Along with this, an analysis of gene expression of collagens showed that collagen III mRNA was upregulated at day 3, whereas its protein was detected elevated at day 14 postburn. Collagen I showed significantly lower protein level at day 7, together with an upregulation of its mRNA on the same time point and followed by restored protein levels similar to control by day 14 postburn. The protein ratio collagen I/III was significantly reduced by day 14. A similar shift in collagen expression at early time points has been observed for tendon-to-bone healing in rotator cuff injury (17). Analysis of gene expression of TGFβ1 showed significant upregulation at day 14. This is an important factor involved in musculoskeletal tissue differentiation that has been reported to correlate with formation of scar tissue and less organized fibers in healing tendon (17). The synthesis of collagen III protein, upregulation of TGFβ1, and the decrease in the collagen I/III protein ratio suggest an induction of scar tissue or remodeling (15). Another proinflammatory cytokine that is upregulated in rheumatic diseases and present in tendon enthesis is IL-17a (24). This is produced by entheseal CD3+ lymphocytes, peripheral blood CD4+ T cells, and entheseal γ/δ T cells. We analyzed its gene expression throughout the time course, but did not find statistical significance (data not shown). This could be explained due to the chronic nature of rheumatic diseases compared with this more acute response.

Histological observations of collagen alignment and tenocyte distributions showed less tissue organization 14 days postburn compared with control. H&E staining showed less fiber alignment, cell aggregations, and even round nuclear morphology on some cells. This, together with the observation of changes in collagen composition, further indicates cellular events for tissue remodeling and possible weakening of the tendon. Collagen distribution and alignment are crucial for the transmission of forces in the tendon (14). In mature tissues, fibrils can be found parallel to the axial direction of tissue and also interwoven with the presence of a crimp pattern that provides its tensile properties (14, 15, 25). This anisotropy will derive into nonuniform strains on the cells, which can alter the cell morphology and response to differential mechanical forces. To address whether molecular changes in tissue structure will also affect its functional mechanics, the tensile force and deformation were measured. Overall, our findings showed that ultimate force and stiffness were reduced at 14 days postburn compared with controls. These biomechanical results correlate with histological findings of less organized fibers. It is possible that the initial loss in collagen I followed by an induction of synthesis of both collagens I and III results in fiber disorganizationbecause collagen III has been previously reported as accumulated at the rupture site in tendon (15). Although previous reports have shown that Achilles tendon

could react to an inflammatory event produced in surrounding tissue (26), this study is the first to report that remote systemic inflammation is capable of inducing a local inflammatory response and remodeling in the tendon. Other models of trauma that generate systemic inflammation, such as fracture hematoma, have reported a similar pattern of circulating cytokines (27–29). Fracture hematoma with additional chest trauma has shown that systemic inflammation is capable of interfering with the normal inflammatory process on fracture healing (30). This evidence is supportive to the hypothesis that a trauma-generated systemic inflammation is potentially detrimental for soft tissues like tendon.

Severe burn patients suffer skeletal muscle and bone mass loss that can be attenuated by the introduction of physical activity. Considering the tendon as the connector and force transmitter between skeletal muscle and bone, it is essential to understand tendon's behavior in response to severe injury. Future investigation regarding the modulation of the inflammatory cascade and MMP inhibition accompanied by regulated physical therapy that allows proper tissue loading to prevent disuse, but also prevent overuse (31–35) may be promising for the improvement of recovery after severe burn and other traumatic injuries.

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Combination Therapies for the Mitigation of Musculoskeletal Pathologic Damage in a Novel Model of Severe Injury and Disuse

OR120033/OR120033P1 Award Number W81XWH-13-1-0489/W81XWH-13-1-0462

PI: Charles Wade, PhD and Steven Wolf, MD **Org:** UT Health, Houston/UT Southwestern, Dallas **Award Amount:** 1,081,066

Study/Product Aim(s)

- Aim 1: Characterize the effect of resistance exercise on muscle and bone health in a validated model of burn and disuse.
- Aim 2: Evaluate the effect of resistance exercise in combination with currently used pharmacological therapies on muscle and bone health in a validated model of burn and disuse.
- Aim 3: Determine the interrelationship between muscle and bone after re-ambulation following pharmacological interventions and exercise.

Approach

A 40% TBSA severe burn will be induced followed by disuse for 14 days. Rats will be assigned to vehicle or drug treatment and further into exercise or no exercise groups. We will examine effects of re-ambulation with or without further resistance exercise after the 14 days. Blood and select organs, muscles and bones will be removed and weighed for testing of mechanical properties, typing, bone morphology, mineral content, and microarchitecture measurements.

Muscle Dimensions and Isometric Muscle Function

Parameter	Group	Plantaris				Soleus			
		No Exercise		Exercise		No Exercise		Exercise	
		Vehicle	Insulin	Vehicle	Insulin	Vehicle	Insulin	Vehicle	Insulin
Muscle	Wet weight (mg)	332 ± 18	329 ± 9	348 ± 6.3	354 ± 14	119 ± 2	143 ± 23	151 ± 32	131 ± 7
	Lo (mm)	35 ± 2	31 ± 0.3	32 ± 1	32 ± 1	33 ± 1.4	29 ± 0.4	31 ± 0.9	30 ± 0.4
	PCSA (mm^2)	27 ± 3	30 ± 2	30 ± 2	#31 ± 2	5.0 ± 0.2	6.9 ± 2.3	6.7 ± 1.3	6.0 ± 0.3
Twitch Force	Pt (g)	89 ± 9	85 ± 3	92 ± 2	‡102 ± 8	10 ± 2	10 ± 2	14 ± 2	*18 ± 1
Tetanic Force	Po (g)	430 ± 31	459 ± 12	508 ± 14	†522 ± 17	38 ± 8	38 ± 9	59 ± 5	*69 ± 5
	Po/CSA (N/cm^2)	16 ± 2	15 ± 2	16 ± 1	17 ± 1	7.4 ± 2	7.0 ± 1	10 ± 2	‡12 ± 1
Pt/Po (%)		21 ± 1	19 ± 1	18 ± 0.4	20 ± 1	26 ± 1	24 ± 2	24 ± 2	26 ± 2
Fatigue	Maximum (g)	——	——	——	——	33 ± 6	34 ± 9	53 ± 4	*64 ± 4
	Minimum (g)	——	——	——	——	27 ± 6	27 ± 6	†46 ± 4	*54 ± 4
	Index (%)	——	——	——	——	81 ± 7	84 ± 5	87 ± 5	84 ± 5

Lo = optimal muscle length
PCSA = Physiological Cross Sectional Area
Po/CSA = Tetanic force normalized to PCSA
Pt/Po (%) = Ratio of twitch to tetanic force
Fatigue Index = Ratio of fatigue minimum to maximum

* vs. No Exercise (ANOVA, p<0.05)
† vs. Vehicle No Exercise (ANOVA, p<0.05)
# vs. Vehicle No Exercise (one-tailed t-test p<0.05)
‡ vs. Other groups combined (two-tailed t-test, p=0.05)

Data summary 1 (for Aim 2): Muscle functions significantly elevated in both plantaris and soleus with insulin and excise combined treatment, wiith increasing Akt/eEF2 protein synthesis pathway and decreasing MuRF-1 ubiquitin protein degradation pathway.

		Plantaris				Soleus			
		VEH-NE	VEH-EX	INS-NE	INS-EX	VEH-NE	VEH-EX	INS-NE	INS-EX
Tissue weight	(g)	0.44540.057	0.4782±0.007	0.484±0.030	0.461±0.040	0.166±0.021	0.177±0.008	0.170±0.023	0.169±0.019
Optimal length	Lo (mm)	39.17±11.82	37.42±16.88	39.67±16.31	36.75±15.88 *	41.75±13.48	40.17±5.98	41.00±26.58	39.00±14.01
Twitch Force	Pt (g)	149.45±17.42	127.64±13.25	149.35±18.13	136.33±21.89 *	58.20±44.47	52.19±21.65	69.02±48.91	52.87±33.38
Tetanic Force	Po (g)	672.11±33.87	613.71±50.31	647.59±67.86	527.93±190.32	180.94±35.71	185.08±34.04	217.33±25.50	224.54±29.02 +
Specific Po	sPo	618.40±118.43	509.68±120.16	549.23±59.18	444.93±183.95	475.12±113.67	437.45±99.75	546.15±65.54	530.88±104.25
	Pt/Po	0.229±0.029	0.210±0.025	0.230±0.014	0.269±0.096	0.326±0.073	0.270±0.023	0.312±0.087	0.249±0.023 *
						Fatigue max (g)	180.4 ± 23.4	187.4 ± 45.6	182.6 ± 25.4	202.1 ± 24.9
						min(g)	13.4 ± 6.3	19.1 ± 14.0	14.2 ± 11.2	22.7 ± 8.2

Mean±SD | *, vs. exercise, p <0.06 | +, vs. insulin, p <0.05

Data summary 2 (for Aim 3): Exercise training decreased plantaris twitch force and optimal length in burn/HLU treated rats. Rat slow-twitch myofiber dominated soleus tetanic force (Po) with insulin treatment increased after re-ambulation period.

Accomplishment: Animal experiments and muscle function test for Aim3 have been completed. Five abstracts and 2 manuscripts were completed in the 4th year period.

Timeline and Cost

Activities / CY	14	15	16	17
Aim 1-Burn/Disuse + Exercise				
Aim 2-Burn/Disuse + Exercise +Pharmacological Therapy				
Aim 3-Burn/Disuse + Exercise +Pharmacological Therapy +Exercise				
Aim 3-Burn/Disuse + Exercise +Pharmacological Therapy +Exercise				
Estimated Budget ($K)	**$399**	**$348**	**$232**	**$101**

Updated: (25 Oct 2017)

Goals/Milestones for Current Award Year

CY14 Goal

- ☑ Obtain all animal approvals and order supplies
- ☑ Aim 1 animal experiment and sample collections
- ☑ Complete muscle function test, uCTs and bone testing
- ☑ Complete ELISAs (In progress)

CY15 Goals

- ☑ Aim 2 animal experiment and sample collections (Study 1-complete; Study 2-in Progress)
- ☑ Complete muscle function test, ELISAs, uCTs and bone testing (In progress)

CY16 Goal

- ☐ Aim 3 animal experiment and sample collections (In Progress)
- ☐ Complete muscle function test, ELISAs, uCTs and bone testing (In Progress)

CY17 Goal

- ▣ Aim 3 animal experiment and sample collections
- ▣ Complete muscle function test,
- ☐ Submission of final results manuscript (In progress)

Comments/Challenges/Issues/Concerns N/A

Budget Expenditure to Date

Projected Expenditure:410,248
Actual Expenditure: 398.908