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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.**

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Nothing listed

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Table of Contents

Page

1. Introduction…… 4
2. Keywords……..4
3. Overall Project Summary……4
4. Key Research Accomplishments…….7
5. Conclusion……7
6. Publications, Abstracts and Presentations……7
7. Inventions, Patents and Licenses……..8
8. Reportable Outcomes……8
9. Other Achievements……8
10. References……..9
11. Appendices……..9

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

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 begin and complete experiments for Aim 2, including animal assay work and data analysis. There were six major tasks associated with this milestone.
Due to major personal translocation in Year 3, we delayed animal experiment schedule for Specific Aim 3 under DOD one year extension approval. The milestone for Year 3 was to continuing data analyzing and reporting.

RESULTS SUMMARY (WOLF):

FOR SPECIFIC AIM 1:

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

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:

- <u>The improvement of exercise and insulin pharmacological combination in rat muscle</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), 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, $p=0.05$)

- <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

4 KEY RESEARCH ACCOMPLISHMENTS

FOR YEAR 3

- Animal experiments for proposed **Aim 2** (Task 3A) were completed.
- (Major task 4) muscle tissue RNA extraction, genomic profiles analysis are complete.
- (Major task 6) is currently on-going. Current data analyzed and four abstracts were presented at Academic Surgical Congress (ASC), American Burn Association (ABA) and Shock Annual Meeting in 2016. One manuscript is published at J Burn Care Res.

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 or are currently on-going. All six major tasks for Year 2 have been completed or are currently on-going.

In both Aim 1 and Aim 2, μCT analysis is currently *on-going* due to change in personnel and availability of the equipment to complete the scanning and analysis of the bones. In both Aim 1 and Aim 2, abstract and manuscript submissions are currently *on-going* as they are actively being drafted and submitted.

Experimental planning for Aim 3 is in progress. The primary focus will be determining the interrelationship between muscle and bone following re-ambulation following pharmacological interventions and exercise.

6 PUBLICATIONS, ABSTRACTS AND PRSENTATIONS

(Abstract/Manuscripts (Appendix 2):

ABSTRACT PRESENTED IN 2016: (2015 Sep to 2016 Aug)

11th Annual Academic Surgical Congress (2016 ASC)

1. **Combined Effects of Insulin and Exercise on Muscle Function in Severe Burn.** Melody R. Saeman[1], Kevin DeSpain[1], Juquan Song[1], Lisa A. Baer[2], Charles E. Wade[2], Steven E. Wolf[1]. [1]University Of Texas Southwestern Medical Center, Surgery, Dallas, TEXAS, USA; [2]University Of Texas Health Science Center At Houston, Surgery, Houston, TEXAS, USA

2016- American Burn Association (ABA) Annual Meeting.

1. **Exercise Treatment Reversed Micro RNA Profile in Burn Rats with Hindlimb Unloading.** A. Cai[1], J. Song[1], P. Kumar[1], A. Sehat[1], M.R. Saeman[1], L.A. Baer[2], C.E. Wade[2], S.E. Wolf[1]. [1]University Of Texas Southwestern Medical Center, Surgery, Dallas, TEXAS, USA; [2]University Of Texas Health Science Center At Houston, Surgery, Houston, TEXAS, USA

2. **Effects of Resistance Exercise and Daily Insulin on Body Mass, Food Intake, Fat Mass and Total Hindlimb Muscle Mass in Rats Following Burn and Disuse.** L.A. Baer[1], J. Song[2], S.E. Wolf[2] and C.E. Wade[1. 1]Center for Translational Injury Research and Dept. of Surgery, University of Texas Health Science Center-Houston, Houston, TX and [2]University of Texas, Southwestern Medical Center, Dallas, TX.

2016- Shock Society Annual Meeting

1. **Combined Effects of Oxandrolone and Exercise on Muscle Function Recovery in Rats with Severe Burn and Hindlimb Unloading.** J. Song[1], K. DeSpain[1], L. A. Baer[2], C. E. Wade[2], S. E. Wolf[1]. [1]University Of Texas Southwestern Medical Center, Surgery, Dallas, TEXAS, USA; [2]University Of Texas Health Science Center At Houston, Surgery, Houston, TEXAS, USA

7. INVENTIONS, PATENTS and LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

Nothing to report.

9. OTHER ACHIEVEMENTS

Nothing to report.

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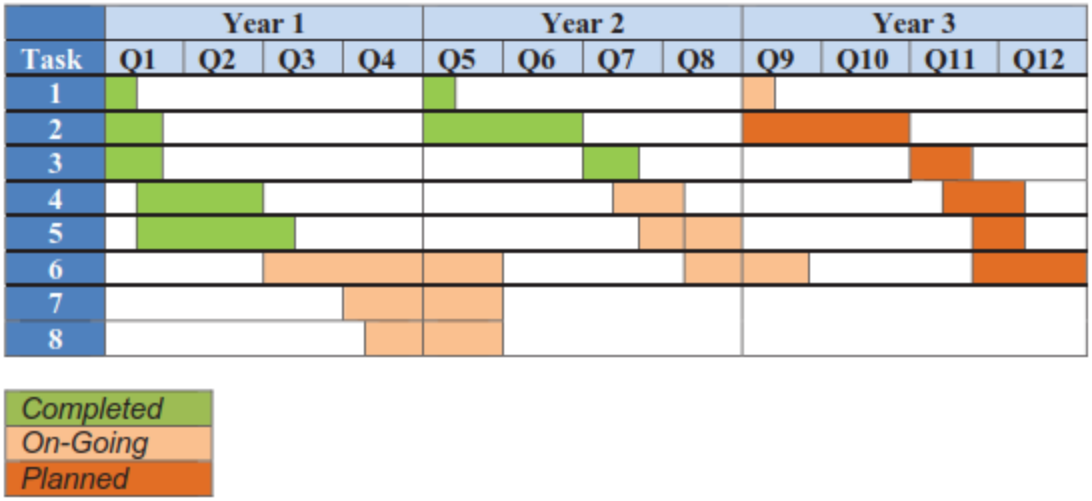
10. Pidcoke HF, Baer LA, Wu X, Wolf SE, Aden JK, Wade CE**.** Insulin effects on glucose tolerance, hypermetabolic response, and circadian-metabolic protein expression

11. APPENDICES

1. Project Timeline
2. Submitted Abstracts/Manuscripts
3. Quad Chart

TASK PROGRESS SUMMARY CHART



ABSTRACT CONTENT (ABSTRACT PRESENTED IN 2016):

Combined Effects of Insulin and Exercise on Muscle Function in Severe Burn

Melody R. Saeman[1], Kevin DeSpain[1], Juquan Song[1], Lisa A. Baer[2], Charles E. Wade[2], Steven E. Wolf[1]

[1]University of Texas Southwestern Medical Center, Dallas, Texas; [2]University of Texas Health Science Center at Houston, Houston, Texas

Introduction:

Muscle loss is a known sequela of severe burn and critical illness that increases the risk of complications such as sepsis and prolonged recovery time. A prior study in a rat model of hindlimb unloading after burn supports that bedrest contributes significantly to muscle atrophy. The aim of our study was to evaluate if exercise combined with insulin in the immediate recovery period mitigates the loss of muscle function in this animal model.

Methods:

Twenty Four Sprague-Dawley rats received a full thickness 40% total body surface area (TBSA) burn and were randomly assigned (n=6) to vehicle without exercise (V/No), insulin (pro zinc 40U daily) without exercise (I/No), vehicle with exercise (V/Ex), or insulin with exercise (I/Ex). All animals were placed in a tail traction system for hindlimb unloading to mimic bed rest immediately following burn. The exercise group was trained to perform twice daily weighted resistance climbing of 1 meter with 5 repetitions. On day 14 *in situ* isometric forces of the left soleus and plantaris muscles were measured. Fatigue measurement was performed in only the soleus. Statistical analysis was performed with Sigma Plot using Student's t-test or ANOVA where appropriate.

Results:

There was no significant change in animal body mass between treatments. The physiological cross sectional area (PCSA) of the plantaris increased with combined insulin and exercise. The

tetanic (Po) and twitch (Pt) muscle functions were significantly elevated in the plantaris of I/Ex. However, there was no change in the tetanic force when normalized to PCSA (Po/CSA). The soleus had significant elevation of Po, Pt, Po/CSA, fatigue maximum, and fatigue minimum in I/Ex. Please refer to the table for specific values and significance.

Conclusion:

Insulin and resistance exercise have a positive combined effect on the hindlimb muscle function in this model of critical illness. The plantaris muscle demonstrated increased physiological cross sectional area with increased force suggesting a net increase in muscle fibers as the cause of this change. The soleus demonstrated a change in the specific force of the muscle and fatigue functions indicating a change in the composition of muscle fiber types.

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, $p=0.05$)

Exercise Treatment Reversed Micro RNA Profile in Burn Rats with Hindlimb Unloading

Anthony Cai[1], Juquan Song[1], Puneet Kumar[1], Alvand Sehat[1], Melody R. Saeman[1], Lisa A. Baer[2], Charles E. Wade[2], Steven E. Wolf[1]

[1]University of Texas Southwestern Medical Center, Dallas, Texas; [2]University of Texas Health Science Center at Houston, Houston, Texas

Introduction:

Micro RNA (miRNA) is a class of non-coding RNA that regulates gene expression by silencing messenger RNA. Burn induces muscle breakdown that is made worse by bed rest, while exercise has been found to alleviate this muscle atrophy. We hypothesize that the alteration of miRNA and target gene profiles contributes to skeletal muscle mass loss after burn, and exercise reverses the muscle atrophy. The purpose of our study was to characterize the miRNA profile correlated to gene expression in an animal model for burn and disuse, as well as miRNA changes seen with exercise.

Methods:

Forty-eight Sprague-Dawley rats were randomly assigned to sham ambulatory (SA), burn ambulatory (BA), sham hindlimb unloading (SH), and burn hindlimb unloading (BH) groups. Rats received a 40% total body surface area scald burn or sham treatment, and they were placed in hindlimb unloading by tail harness, a model for bed rest, or ambulatory. Half of each group received twice daily resistance exercise for eight total groups (n=6 per group). After the 14-day treatment period, the plantaris muscles were harvested for miRNA and genomic data analysis.

Results:

Our results show that compared to the SA group, BA and SH independently upregulate 3- to 4-fold more miRNAs and genes than they downregulate. miRNA-182, -187-3p, and -155-5p rank among the most upregulated. Comparing the combination of B and H (BH) to SA reveals that miRNA-182, miRNA-187-3p, and gene Nr4a3 receive additive contributions from B and H. With exercise, miRNA-182 increased 10.06-fold, and miRNA-138-1-3p decreased 2.14-fold. In comparison, exercise in the BH group strongly downregulated miRNA-182 7.04-fold and miR-138-1-3p 6.57-fold. In a similar fashion, exercise upregulated genes Chad and Cpxm2 in SA, then downregulated them in BH. [Table 1]

Conclusions:

Burn and disuse additive contributions to miRNA and gene changes may explain the additional muscle atrophy burn patients experience with bed rest. Furthermore, exercise demonstrates a greater downregulation of miRNA and genes in BH compared to the SA group.

Applicability of Research to Practice: Elucidating specific miRNAs' roles in muscle atrophy secondary to burn and bed rest opens the possibility of new markers and treatments targets.

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

Table 1. miRNA and gene expression changes in response to B, H, BH, exercise in SA, and exercise in BH.

miRNA/Gene	BA vs. SA	SH vs. SA	BH vs SA	SA: Exercise vs. No Exercise	BH: Exercise vs. No Exercise
miR-138-1-3p	n/a	9.05	14.7	-2.14	-6.57
miR-155-5p	8.82	9.47	7.81	5.66	n/a
miR-187-3p	4.19	4.24	7.9	6.15	n/a
miR-182	12.81	23.83	35.35	10.06	-7.04
mir-184	10.5	17.73	n/a	3.34	2.13
Nr4a3	2.56	3.59	6.31	n/a	-2.38
Cpxm2	2.63	n/a	2.7	2.08	-2.49
Fmod	2.64	n/a	2.77	n/a	-3.01
Chad	6.22	n/a	4.52	3.91	-2.44

---- n/a: fold change is lower than criterion threshold of 2

Effects of Resistance Exercise and Daily Insulin on Body Mass, Food Intake, Fat Mass and Total Hindlimb Muscle Mass in Rats Following Burn and Disuse

L.A. Baer[1], J. Song[2], S.E. Wolf[2] and C.E. Wade[1]
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Introduction: Treatment and recovery of patients with severe traumatic injuries is impacted by an increase in metabolism. Injury induces a systemic catabolic response with increased energy expenditure and loss of body mass. After burn injury, the ability to resume normal activities is compromised due to inactivity associated with bed rest as well as the catabolic response. Exercise and nutritional interventions have been used independently with limited success. The purpose of this study was to determine following burn and disuse how body mass, food intake and total hindlimb muscle mass are affected by a combination of daily resistance exercise and daily insulin.
Methods: Male, Sprague-Dawley rats were randomized into four groups: Burn/Hindlimb unloaded (BH) with daily resistance exercise (EX) or no exercise (NEX) and vehicle control (VEH) or insulin-treated (INS). Daily resistance exercise began ten days prior to injury by adding weight to the tail during repetitive ladder climbing. Rats were then weight-matched into treatment groups, either daily exercise or no-exercise, VEH or INS. Body mass, food intake was collected daily throughout the study. Fat mass and total hindlimb muscle (TA+EDL+Plantaris+Soleus+Medial and Lateral Gastroc) mass was measured on D14. Data are mean±SEM and an ANOVA was used with significance at $p<0.05$.
Results: No differences in body mass were observed between any groups at the time of injury or day 14. Exercise caused a significant increase in mean food over the last 5 days independent of daily insulin. Fat mass was not different between any of the groups. Exercise significantly increased total muscle mass irrespective of insulin. Total hindlimb muscle mass was further increased with the combination of daily insulin and exercise.
Conclusions: Exercise independently and with insulin elicited a significant increase in food. However, the increase in food intake did not result in differences between treatment groups in either body mass or fat mass, supporting a hypermetabolic effect. Muscle wasting was reduced with daily exercise. When daily exercise was combined with daily insulin, there was a greater attenuation of muscle wasting, suggesting the combination of exercise and insulin may be a contributing factor in reversing wasting as a result of burn injury. Different underlying factors seem to be influencing the acute metabolic changes offering possible opportunities for combinations of early interventions resulting in positive long-term outcomes.
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	BH-VEH	BH-INS
D14 Body Mass (g) (n=63)		
NEX	270±3	271±4
EX	273±3	262±6
Food Intake (Last 5 days-g/100g BM/day) (n=63)		
NEX	7.2±0.1	7.3±0.2
EX	7.4±0.1*	7.8±0.2*
Fat Mass (per 100g BM) (n=63)		

NEX	0.62±0.03	0.69±0.04
EX	0.66±0.03	0.65±0.05
Total Hindlimb Muscle Mass (per 100g BM) (n=63)		
NEX	0.88±0.01	0.90±0.02¥
EX	0.95±0.01*	0.99±0.02*¥

Mean ± SEM; *p<0.05 from NEX ¥ p<0.05 from VEH

Combined Effects of Oxandrolone and Exercise on Muscle Function Recovery in Rats with Severe Burn and Hindlimb Unloading

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Introduction: Muscle mass loss and function impairment worsens with restricted mobility in severe burn patients. We previously showed exercise training improved muscle function recovery in severely burned rats with hindlimb unloading. The aim of the study was to evaluate if the combination of daily oxandrolone with resistance exercise mitigates the loss of muscle function in this animal model.

Methods: Twenty Four Sprague-Dawley rats received a full thickness 40% total body surface area (TBSA) burn and were randomly assigned to vehicle (corn oil) without exercise (V/NEX), oxandrolone (0.1mg/kg/day) without exercise (O/NEX), vehicle with exercise (V/Ex), or oxandrolone with exercise (O/Ex) (n=6/group). All animals were placed in a tail traction system for hindlimb unloading to mimic bed rest after burn. The exercise groups completed resistance training twice a day. On day 14 *in situ* isometric forces of the left plantaris and soleus muscles were measured by using the ASI muscle lever system with dynamic muscle control and analysis software (Aurora Scientific, Inc). Fatigue measurement was only performed in the soleus. Statistical analysis was performed with Sigma Plot using Student's t-test or ANOVA where appropriate.

Results: Tetanic (Po) muscle function were significantly elevated in the plantaris with exercise ($p=0.038$), but not with oxandrolone treatment. Fatigue index (FI) was lower and integration significantly elevated in the soleus due to exercise ($p<0.05$) irrespective of oxandronlone treatment [Table1].

The generalized estimating equation linear regression model was applied to further analyze data during a 240s observation period of the fatigue curve in rat soleus. Results showed that the average max force were achieved in rat soleus either with oxandrolone treatment or resistance exercise independently. The max force for the O/Ex group was significantly higher than that for the V/NEX group ($p=0.01$).

Conclusion: Resistance exercise improves muscle function in plantaris and soleus in burn rats with hindlimb unloading, and that improvement is irrespective of oxandrolone treatment.

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Table 1

	NEX		Ex			NEX		Ex	
Plantaris	V	O	V	O	Soleus	V	O	V	O
Tissue weight (g) /100g BM	0.119±0.015	0.133±0.013	0.122±0.019	0.133±0.011	Tissue weight (g)/100 g BM	0.028±0.010	0.032±0.006	0.034±0.003	0.037±0.006
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	26.4 ± 11.8%	27.2 ± 13.0%	15.6 ± 7.7%	16.1 ± 6.4%*
Po/Pt	4.29 ± 1.01	4.17 ± 0.70	4.48 ± 0.94	5.31 ± 0.55	Fatigue (max)	79.89 ± 45.63	95.075 ± 36.44	100.25 ± 13.55	104.71 ± 11.34
sPt (N/cm2)	6.79 ± 0.80	8.97 ± 2.97	7.85 ± 1.45	8.19 ± 1.02	Integration	4837.4±2608.6	4830.8±2371.4	6614.2±1864.5	7985.4±727
sPo (N/cm2)	29.69 ± 10.06	35.212 ± 7.44	34.733 ± 6.98	38.25 ± 11.78	sPt (N/cm2)	9.69 ± 5.55	10.81 ± 4.50	14.05 ± 8.33	9.52 ± 1.74
					sPo (N/cm2)	22.67 ± 14.04	29.08 ± 13.79	23.41 ± 6.09	24.46 ± 4.14

∗, p<0.05, exercise vs. non exercise, two way ANOVA , value presented as mean±SD

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

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Abstract

Objective—This study investigated microRNA and target gene profiles under different conditions of burn, bed rest, and exercise training.

Methods—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% total body surface area 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.

Results—Compared to the sham ambulatory-no exercise group, 73, 79, and 80 microRNAs were altered two-fold in the burn ambulatory, sham hindlimb unloading, and burn hindlimb unloading groups, all with no exercise, respectively. Over 70% of microRNAs were upregulated in response to burn and hindlimb unloading, while 60% microRNA of the profile decreased in hindlimb unloaded burn rats with exercise training. MiR-182 was the most affected in rat muscle. GO 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.

Conclusions—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.

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Author contributions: JS, MRS, and ARC contributed to data collection and analysis and manuscript preparation. LAB was for animal experiment, sample collection, and manuscript revision. CEW and SEW were integral to the concept and design of the experiment as well as the critical revision of the manuscript.

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Keywords

epigenetics; transcriptome; thermal injury; bed rest; exercise

Introduction

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 upon 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 bi-phase 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%-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 PTSD 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. previously studied the miRNA profile of cells in burned dermis and found 66 miRNAs that were significantly up- or downregulated.[8] 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 demonstrated that rat muscle function decreased after burn and hindlimb unloading in an animal model.[10] We speculated that miRNA changes in skeletal muscle after burn and bed rest contribute to muscle atrophy due to 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 carried out at UT 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% total body surface area under 2%-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 meter at an 80-degree 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 non-exercised group (N) were pre-trained 10 days prior to injury.

Total RNA extraction and genomic analysis

On day 14 after injury, all animals were euthanized, 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 3 animals in each group were pooled, and total RNA was extracted using Qiagen miRNeasy Mini Kit (Qiagen, Hilden, Germany). RNA purity was over 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 (intra and inter-lot) is greater than 0.95. A total of

36,333 small non-coding RNA probes, including varied species and controls 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 (Santa Clara, CA). The chips contains 30,429 rat gene probes. Raw signal intensity data was normalized with robust multi-array average (RMA) 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 2. For triplicated genomic sample data analysis, a one-way between-subject ANOVA (unpaired) was further applied with significant acceptance of $p < 0.05$. 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 2 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 2. There were 73, 79 and 80 miRNAs altered in the B/A/N, S/H/N, and B/H/N groups, respectively, compared to S/A/N group. Over 70% of the miRNAs were upregulated in response to burn and hindlimb unloading, while about 60% of miRNAs were upregulated in B/H rats with exercise training (Figure 2 A).

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, while 239 genes were disturbed in combined burn and hindlimb unloading rats. There were 2, 20, and 22 genes that increased greater than fourfold in the B/A, S/H, and B/H groups separately. In contrast, 62 out of 71 genes decreased in the B/H/E versus B/H/N groups (Figure 2 B).

MiRNA and gene expression profiles are distinguished in response to burn, hindlimb unloading, and exercise and described separately as follows:

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 miRNAs was higher than that of downregulated ones. There were 14 miRNAs upregulated more than fourfold, including the 3 most upregulated miRNAs, miR-182 (12.81), miR-184 (10.50) and miR-155-5p (8.82). Fold changes were less than 3 in all 15 downregulated miRNAs. The miR-409a-3p was the most decreased (-2.95) in burn animals (Supplemental Table 1-1).

There were 47 genes changed, 12 down- and 35 upregulated, in the B/A/N group compared to the S/A/N group (Supplemental Table 1-2). Thirty-two changed genes were associated with multiple GO biological processes, and 6 signal pathways were altered. The ketone body metabolism, inflammatory response, and striated muscle contraction pathways were most activated (Supplemental Table 1-3).

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 over fourfold, and 10 miRNAs even changed over eight-fold; in contrast, downregulated miRNAs were all changed less than fourfold. 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) (Supplemental Table 2-1).

There were 135 genes, 100 up- and 35 downregulated, in rat muscle in response to hindlimb unloading. In all, 20 genes increased more than fourfold; only 2 genes decreased more than fourfold (Supplemental Table 2-2). Seventy-nine genes had varied biological process functions involving 9 pathways, with the 3 most prominent pathways being the MAPK cascade, the blood-clotting cascade, and fatty acid synthesis (Supplemental Table 2-3).

In viewing the interaction network, a large complex network was constructed between miRNAs 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 -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 3 downregulated over fourfold. There were 17 miRNAs upregulated over fourfold, and miR-182 even increased 35-fold (Supplemental Table 3-1).

There were 239 genes altered between the B/H/N versus S/A/N group. Only 3 of 115 downregulated genes changed more than fourfold, whereas there were 22 out of 124 genes upregulated more than fourfold (Supplemental Table 3-2). 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 (Supplemental Table 3-3).

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 to 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; the *Mbp* gene increased in an opposite manner; while Nr4a3 had an additive effect in response to the combination of burn and hindlimb unloading (Supplemental Table 4).

The miRNA and gene expression profile in rats with burn and hindlimb unloading 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 9 miRNAs downregulated over fourfold, while 7 were upregulated over fourfold. The most upregulated miRNAs include miR-1843-3p (8.52), miR-495 (5.92), and miR-6324 (5.78) (Supplemental Table 5-1).

In viewing the interaction network, there were 71 gene changes, with 62 downregulated genes and only 9 genes upregulated less than fourfold in transcriptional level (Supplemental Table 5-2). Overall, 54 genes partook in different biological processes and 7 pathways. The MAPK cascade, fatty acid synthesis, and the inflammation response pathway were the top 3 alleviated pathways (Supplemental Table 5-3).

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 down- and 55 upregulated. We found that miR-182 increased 10.06-fold in S/A/E. There were only 12 genes altered, with 7 upregulated and 5 downregulated. Though 9 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. Hyper-metabolic 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. 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.[16] Vemula et al. verified this, linking 28% of the changed genes to metabolism. These included genes responsible for triglyceride utilization, fatty acid import, and acute phase proteins.[17] Merritt reported that the inflammatory response activated stat/NFκβ to calcium-mediated proteolysis and ubiquitin-proteasome with absence of protein synthesis inhibition.[18] In addition, we found that muscle mass loss is 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]. Though 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's previous report that the hypermetabolic status can even last for years in burn patients.[20]

In human patients with bed rest, a gene profile reveals changes in energy pathways: oxidative phosphorylation, TCA cycle, organic compound usage, and carbohydrate metabolism.[21] Bonaldo and Sandri revealed the intracellular mechanism by which hindlimb unloading activates cell apoptosis in an NF-KB dependent manner.[22] 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 reported that the injection of synthesized miR-182-based spherical nucleic acids suppressed tumor glioma burden and increased animal survival.[29] 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 microRNA 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 hyper-inflammation 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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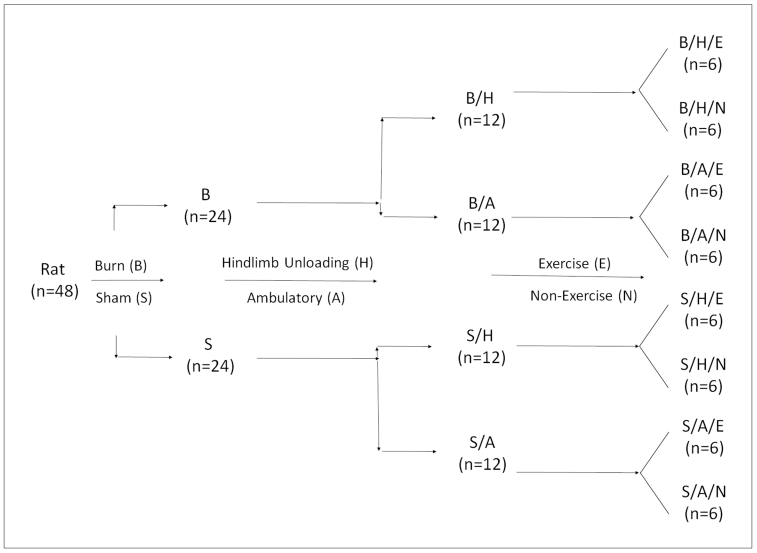

Figure 1.
Flow chart of animal experiment that outlines the size of animal and the order of operation

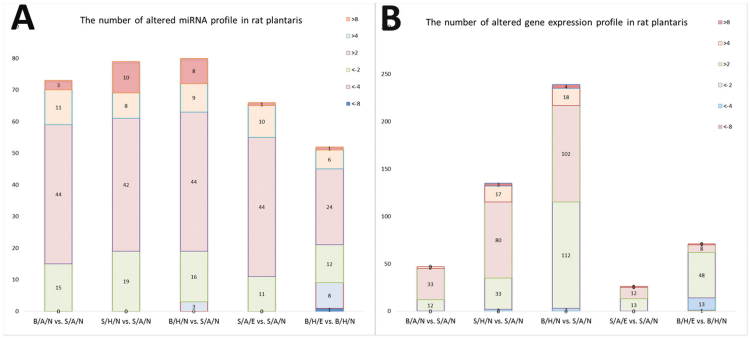

Figure 2.

Stacked bar figures showing the number of miRNA (2A) and genes (2B) 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).

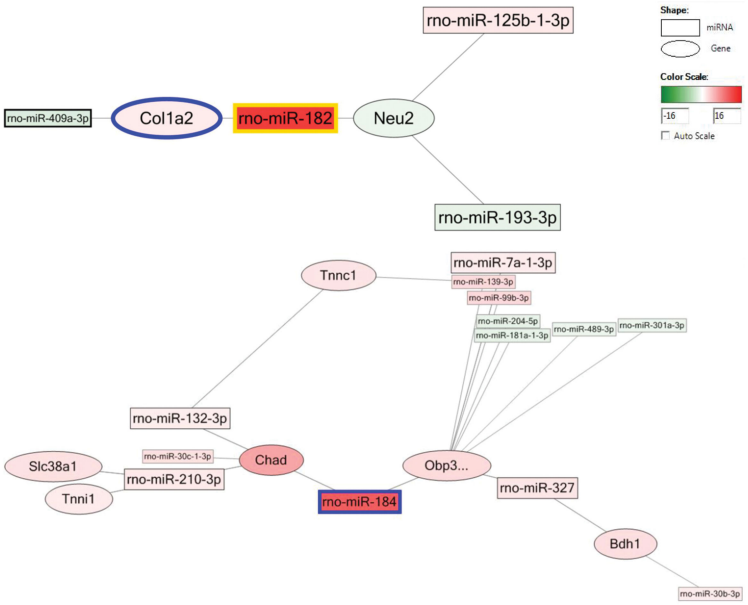

Figure 3.

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

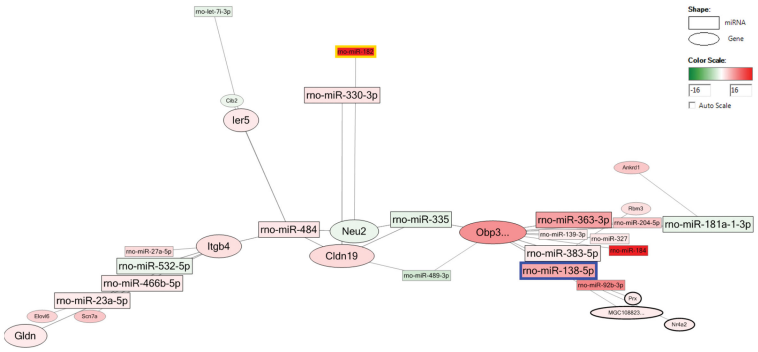

Figure 4.

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

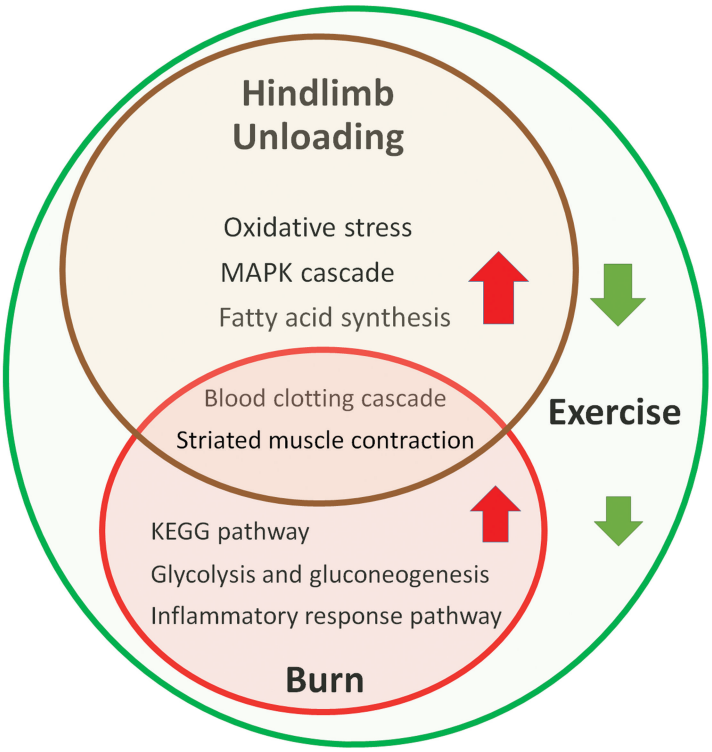

Figure 5.
The scheme of affected signal pathways in response to burn, hindlimb unloading and exercise training. Red arrows indicated activated pathways. Green arrows indicates inhibited pathways.

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		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)

Current data summary 1: Muscle functions including tetanic (Po) and twitch (Pt) were significantly elevated in both plantaris and soleus with insulin and excise combined treatment.

	BH-VEH	BH-INS
D14 Body Mass (g) (n=63)		
NEX	270±3	271±4
EX	273±3	262±6
Food Intake (Last 5 days-g/100g BM/day) (n=63)		
NEX	7.2±0.1	7.3±0.2
EX	7.4±0.1*	7.8±0.2*
Fat Mass (per 100g BM) (n=63)		
NEX	0.62±0.03	0.69±0.04
EX	0.66±0.03	0.65±0.05
Total Hindlimb Muscle Mass (per 100g BM) (n=63)		
NEX	0.88±0.01	0.90±0.02*
EX	0.95±0.01*	0.99±0.02*†

Current data summary 2: Muscle wasting was reduced with daily exercise. When daily exercise was combined with daily insulin, there was a greater attenuation of muscle wasting, suggesting the combination of exercise and insulin may be a contributing factor in reversing wasting as a result of burn injury.

Accomplishment: The experiments for Aim1 and Aim2 have been completed. Six abstracts and 1 manuscript were completed in the 3rd year period. The experiments for Aim 3 are in progress

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: (11 July 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)
- ☐ Submission of final results manuscript

Comments/Challenges/Issues/Concerns

- Due to the key personal relocation, the experiment schedule extended a year

Budget Expenditure to Date

Projected Expenditure:$752,910
Actual Expenditure: $466,652