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TITLE: The Effect of Hypobarica on Muscle Inflammation and Regeneration After Injury and Hemorrhagic Shock

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<b>14. ABSTRACT</b> The purpose of this research is to understand the effect of long-distance flying on recovery after muscle injury and significant bleeding. This situation may affect recovery following combat injuries, especially if wounded service members are traveling from Asia to the United States. The hypothesis, there will be no difference in well-being and white blood cells populations in skeletal muscle between male mice exposed to hypobarica for 16 hours and male mice exposed to normobarica, was supported. In addition, several procedures involved in comparing the effects of two types of resuscitative fluids on muscle recovery and widespread inflammation after significant bleeding were completed. We began procedures to test the hypothesis that long-distance flying at 24 hours after muscle injury and significant bleeding will lead to slower muscle recovery.				
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## 1.0 INTRODUCTION

En route care is a critical and successful strategy for the early intervention of life-threatening battle injuries. Wounded warfighters typically receive en route care during the flight from the battlefield to the nearest medical facility. The issue with this process is the cabin of a medical transport plane is pressurized to 8,000 feet, meaning wounded service members in transit and without supplemental oxygen are exposed to less oxygen than if transported by ground. Therefore, this project aims to examine patient recovery after prolonged exposure to low oxygen levels; identify the effects of exposure; and determine appropriate countermeasures and treatments, if necessary. Laboratory mice will be used to determine exposure effects because their genetic and biological processes mirror those of humans. This approach will allow an adequate assessment of recovery without harming human patients.

Three hypotheses guide this project:

1. Mice exposed to atmospheric pressure equivalent to that of an airplane cabin—i.e., hypobaria—will have muscle-based (intramuscular) white blood cell (WBC or leukocyte) numbers and activity similar to mice exposed to normal atmospheric pressure.
2. Mouse muscle recovery and systemic inflammatory status will vary, depending on the type of resuscitation fluid used after undergoing crush muscle injury and hemorrhagic shock.
3. Hypobaria starting 24 hours after crush muscle injury and hemorrhagic shock with fluid resuscitation will lead to slower muscle recovery.

## 2.0 KEYWORDS

Aeromedical evacuation  
 En route care  
 Hemorrhagic shock  
 Hypobaria  
 Inflammation  
 Skeletal muscle injury  
 Skeletal muscle regeneration

## 3.0 ACCOMPLISHMENTS

### 3.1 Major Year 03 Project Goals

The status of the major Year 03 project goals is summarized in Table 1. Please note that on 23-March-2018, Science Officer Dr. Daphtary requested changes in the statement of work, which are reflected in Table 1. Accomplishment details are provided in Section 3.2.

Table 1. Major Year 03 Project Goals

	Month Completed	% Completed
<b>Specific Aim 1, Major Task 4: Hypotheses 1 and 2 Testing</b>		

Subtask 5: Perform Hypothesis 2 analysis.		80%
Subtask 6: Review Hypothesis 1 and 2 results.		80%
Subtask 7: Write and/or review report.		80%
Subtask 8: Disseminate results.		50%
Subtask 9: Submit and receive animal protocol amendment approval.		95%
Milestone Achieved: Hypothesis 1 and 2 testing and Animal Amendment Approval completed.		80%
<b>Specific Aims 2 and 3, Major Task 1: Hypothesis 3 Testing – C57BL/6 male mice 1–190</b>		
Subtask 1: Complete animal procedures.	May 2019	100%
Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and real time-polymerase chain reaction (RT-PCR) assays (including data entry) for 190 mice.		30%
Subtask 3: Hire, orient, and train laboratory technician.	November 2018	100%
Milestone(s) Achieved: Mice 1–190 testing		60%
<b>Major Task 2: Hypothesis 3 Testing – C57BL/6 male mice 191–380</b>		
Subtask 1: Complete animal procedures.		39%
Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (including data entry) mice.		0%

### 3.2 Accomplishment Details

#### 3.2.1 Subtask 5: Perform Hypothesis 2 analysis.

##### 3.2.1.1 H2B Animal Procedures

As indicated in previous reports, Hypothesis 2 (H2) is divided into H2A and H2B experiments. The H2A experiment was completed in Year 02. The H2B experiment began in Year 02, but analysis was not completed.

The H2B animal procedures began on 16-April-2018 and were completed on 22-June-2018. With this cohort, 67 out of 77 (Table 2) mice survived and underwent tissue analysis. However, because of the attrition experienced and our a priori statistical power analysis, we requested and received approval for additional mice to have a target of 10 mice per group for analysis. Between 18-October-2018 and 14-December-2018, an additional 32 mice were tested. Of these mice, eight were not usable (Table 2). In total, 91 mice were usable from the two cohorts (Table 3). The H2B hypothesis is mice that undergo crush muscle injury and hemorrhagic shock, followed by lactated Ringer's solution (LRS) fluid resuscitation, will demonstrate a slower muscle recovery and greater systemic inflammation than mice that undergo crush muscle injury and hemorrhagic shock, followed by Hextend® (HEX) resuscitation.

Table 2. Updated H2B Completion and Survival Rates

Event	Number <sup>a</sup>	Percent <sup>a</sup>
Starting total	109	NA
Comorbidities discovered after crush injury but before femoral artery catheterization	107	NA
Successful femoral artery catheterization	104 of 107	97
Sham completion <sup>b</sup>	31 of 32	97
Sham survival <sup>c</sup>	31 of 31	100
Shock completion <sup>b</sup>	68 of 72	94
Early death after shock due to known or unknown cause	6 of 68	9
Shock survival <sup>c</sup>	62 of 68	91
Comorbidities discovered during euthanasia	2 of 62	3

Notes. NA = not applicable.

<sup>a</sup>The two mice transferred from H2A are included in these data.

<sup>b</sup>Completion = The mouse underwent crush injury followed by sham hemorrhagic shock or hemorrhagic shock/fluid resuscitation as described in the animal protocol and recovered from anesthesia without experiencing major respiratory problems.

<sup>c</sup>Survival = The mouse was euthanized at the assigned time point.

Table 3. Updated H2B Group Sizes

EU Time Point Days	Sham	HEX	LRS	Total Number
1.5	10	10	10	30
4	11	10	10	31
8	10	10	10	30
Total Number	31	30	30	91

Notes. EU = euthanasia; HEX = Hextend; LRS = lactated Ringer's solution. All mice underwent crush injury of the right gastrocnemius and quadriceps muscles as well as femoral artery catheterization. The mice in the LRS and HEX groups underwent hemorrhagic shock and resuscitation with the corresponding fluid. Mice in the Sham group remained anesthetized for a similar time period as the other mice but did not undergo hemorrhagic shock or fluid resuscitation.

### 3.2.1.2 Body Weight

Figure 1 shows the mean body weight at euthanasia of the mice in the nine groups. The body weight of HEX and LRS mice at 1.5 days was significantly lower than all groups at 4 and 8 days. There were no significant differences in body weight among the three groups at 1.5 days.

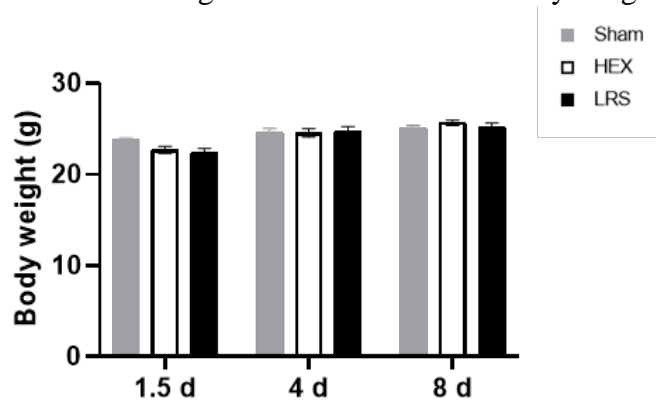


Figure 1. Body Weight at 1.5, 4, and 8 Days Postinjury. The body weight of HEX and LRS mice at 1.5 days (d) is significantly lower than all groups at 4 and 8 d; Values = mean  $\pm$  SE.  $n = 9-11$ .

### 3.2.1.3 Crush Injury

At the time of crush injury, the age and body weight range of the mice was 10–22 weeks and 23–29 g, respectively. The crush stimulus consisted of 45 psi for the majority of mice. For six mice, the crush stimulus was 40, 44, or 50 psi. In 95% of the mice, the gastrocnemius muscle received two applications, and the quadriceps muscle received three applications.

We evaluated the occurrence of hematomas in crush-injured gastrocnemius ( $n = 90$ ) and quadriceps femoris ( $n = 90$ ) muscles (Table 3). Hematomas were present at 1.5 and 4 days postcrush, and occurrence decreased at 8 days postcrush in all groups. Although in the first cohort we observed that the hematomas in the injured gastrocnemius muscles of HEX mice tended to be darker in color than those in the LRS or Sham mice, this observation was not evident in cohort 2 (data not shown).

Table 4. Updated H2B Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Femoris Muscles

EU Time Point Days	Crush-injured Gastrocnemius			Crush-Injured Quadriceps Femoris		
	Sham	Hex	LR	Sham	Hex	LR
1.5	100%	100%	100%	90%	100%	90%
4	100%	100%	100%	90%	100%	91%
8	70%	70%	44%	40%	56%	50%

*Notes.* EU = euthanasia; HEX = Hextend; and LRS = lactated Ringer's solution. Hematomas were assessed by viewing digital images of muscles after harvest. However, images for two muscles were inadvertently not captured.

### 3.2.1.4 Sham Hemorrhagic Shock or Hemorrhagic Shock/Fluid Resuscitation

Table 4 shows that we achieved an adequate completion rate of hemorrhagic shock/fluid resuscitation and an adequate survival rate after fluid resuscitation. Table 5 provides more details regarding the 91 mice that underwent sham hemorrhagic shock or hemorrhagic shock/fluid resuscitation.

Table 5. Other Details Regarding Sham Hemorrhagic Shock and Hemorrhagic Shock/Fluid Resuscitation

Group	Body Weight (g)	BMAP (mm Hg)	Blood Withdrawal (ml)	HS MAP (mm Hg)	FR Volume (ml)	Final MAP (mm Hg)
Sham	25.55 ± 1.19	70.61 ± 5.58	NA	NA	NA	71.19 ± 6.94
HEX	25.11 ± 1.13	70.73 ± 6.14	0.54 ± 0.11	37.93 ± 4.92	1.28 ± 0.35*	62.23 ± 5.55*
LRS	25.43 ± 1.18	68.73 ± 6.13	0.55 ± 0.13	39.47 ± 4.40	0.93 ± 0.47	67.27 ± 3.61*

*Notes.* BMAP, baseline mean arterial pressure; HS, hemorrhagic shock; FR, fluid resuscitation; HEX = Hextend, LRS = lactated Ringer's solution, NA = not applicable. Values = mean ± SD,  $n = 30$ – $31$ . \* = LRS vs. HEX,  $p < .05$  (preliminary analysis).

### 3.2.1.5 Hematological Parameters

Since our consultant recommended the use of the Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter, Atlanta, GA) to generate whole blood leukocyte data, this analysis included other hematological parameters from whole blood: red blood cell (RBC) count, hemoglobin

level, and mean corpuscular volume to calculate hematocrit. We analyzed up to 24 mice using heparinized whole blood (Table 6). Although groups sizes were small, the Sham group values were much lower than the reported values for C57BL/6 mice (even though Sham mice undergo crush muscle injury). Raabe, Artwohl, Purcell, Lovaglio, and Fortman (2011) determined that anemia for male mice is a hemoglobin level < 13.6 g/dl, and severe anemia is 7.3–9.0 g/dl. Based on this definition and the Table 6 data, the possibility exists that the crush injury contributes to anemia and the addition of hemorrhagic shock results in a more severe case of anemia.

Table 6. H2B Hematological Parameters

	RBC 10 <sup>6</sup> /μl	Hgb g/dl	Hct %	<i>n</i>
C57BL/6 males <sup>a</sup>	10.40	15.80	53.70	NA
Sham 1.5 days	8.96	13.33	39.80	2–3
HEX 1.5 days	5.39	7.52 <sup>b</sup>	27.19	1–2
LRS 1.5 days	3.75	5.54 <sup>b</sup>	Not done	2
Sham 4 days	7.99	12.13 <sup>d</sup>	Not done	2–3
HEX 4 days	4.87	9.18 <sup>c</sup>	25.33	1–2
LRS 4 days	5.79	8.46 <sup>b</sup>	28.05	1–2
Sham 8 days	8.70	13.87	38.92	2
HEX 8 days	6.63	10.11 <sup>c</sup>	35.17	3–4
LRS 8 days	7.89	12.14 <sup>d</sup>	37.16	3–4

Notes. RBC = red blood cells, Hgb = hemoglobin, Hct = hematocrit, *n* = group sizes, NA = not applicable, HEX = Hextend, LRS = lactated Ringer's solution.

<sup>a</sup> Reference: <https://www.envigo.com/resources/data-sheets/c57bl6nhsd-male-cbc.pdf>.

<sup>b</sup>Severe, <sup>c</sup>moderate, and <sup>d</sup>mild anemia according to Raabe et al. (2011).

Reference: Raabe, B. M., Artwohl, J. E., Purcell, J. E., Lovaglio, J., & Fortman, J. D. (2011). *Journal of the American Association for Laboratory Animal Science*, 50, 680–685.

### 3.2.1.6 Systemic Inflammation

In the proposal, this dependent variable is divided into two indicators: whole blood leukocyte populations and serum concentrations of proinflammatory cytokines, anti-inflammatory cytokines, and chemokines. However, during the performance of H2A mouse procedures, we observed that spleen weights in certain shock mice were higher than normal. Because an increased spleen weight can reflect systemic inflammation, we added this indicator to our analysis. The following three subsections discuss the accomplishments of these three indicators of systemic inflammation.

#### 3.2.1.6.1 Whole Blood Leukocyte Populations

With the second cohort of mice, we did not perform additional assays with whole blood leukocyte populations for three reasons. First, data from the first cohort did not seem to indicate any strong trends. Second, we needed to focus our attention on other parameters. Third, during Year 03, Quarter 1, the consultant recommended that we use the Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter) to generate whole blood leukocyte data, which would limit reagent costs and reduce processing time. With the second cohort of mice, we switched from whole blood flow cytometry assays to diff2 assays. Using these mice, we tested up to 24 mice using

heparin as an anticoagulant. However, we found that the heparinized blood yielded inconsistent blood leukocyte data.

The consultant's two other recommendations regarding the variable, whole blood leukocyte population, were to (a) perform an ex vivo experiment to determine whether blood monocytes are being primed (to be prepared for activation) by LRS and (b) increase the group sizes. We did not have time to perform recommendation (a). Regarding recommendation (b), we increased the group sizes to reach 10–11 per group as indicated in Table 4, but we did not reach the recommendation of 16 mice per group.

### 3.2.1.6.2 Serum Concentrations of Proinflammatory Cytokines, Anti-inflammatory Cytokines, and Chemokines

We performed multiplex assays to detect inflammatory-related cytokines in serum. Because we had to perform multiple assays and blood was allocated for both multiplex and flow cytometry assays, certain variables assays have low group sizes.

Figure 2 shows the findings for serum erythropoietin and TIMP-1. Significantly higher median EPO levels were detected in the shock mice (HEX and LRS combined) compared to the Sham mice at 1.5 and 4 days postinjury. Another major finding was that higher median TIMP-1 levels were observed in HEX or Shock (HEX and LRS combined) mice than in Sham mice at 8 days postinjury. In addition, we found the median serum level of exodus-2 was significantly higher in the LRS group than in the Sham group at 1.5 days (7,659 pg/ml vs. 3,340 pg/ml,  $p < .05$ ).

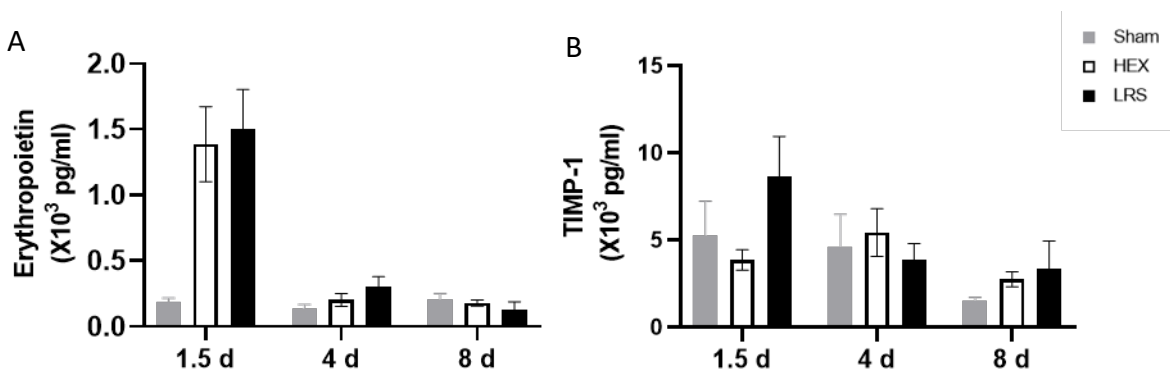


Figure 2. Serum Erythropoietin and Tissue Inhibitors of Metalloproteinases 1 (TIMP-1) Levels at 1.5, 4, and 8 Days Postinjury. Values = mean  $\pm$  SE. However, median values were tested using the Wilcoxon rank sum test (Mann-Whitney U test) for each cytokine at each time point. Erythropoietin, Sham versus Shock (HEX and LRS combined) at 1.5 and 4 days postinjury,  $p < .05$ . TIMP-1, Sham versus HEX at 8 days postinjury,  $p < .05$ .

Another panel consists of three cytokines: granulocyte-colony stimulating factor (G-CSF), leptin, and follistatin. Table 7 lists the serum median levels of these cytokines among the nine groups. Although the serum median level of G-CSF is highest in the HEX group at 1.5 days, no significant difference was detected between the Sham or LRS groups. At 8 days postinjury, the HEX group has the highest median serum leptin level but the lowest serum follistatin level; however, the HEX values were not significantly different from the Sham or LRS groups.

Table 7. Serum G-CSF, Leptin, and Follistatin Levels

Group	G-CSF	Leptin	Follistatin
Sham 1.5 days	907.79	635.48	680.86
HEX 1.5 days	1575.50	341.10	954.55
LRS 1.5 days	932.91	440.99	627.91
Sham 4 days	341.30	782.50	854.54
HEX 4 days	563.64	593.00	882.44
LRS 4 days	519.86	840.85	680.86
Sham 8 days	299.52	613.14	855.52
HEX 8 days	327.00	837.27	436.82
LRS 8 days	314.19	655.02	833.38

Notes. G-CSF, granulocyte colony stimulating factor, HEX = Hextend, LRS = lactated Ringer's solution. Values = median, pg/ml. G-CSF,  $n = 5-9$ ; leptin,  $n = 5-9$ ; follistatin,  $n = 3-9$ .

### 3.2.1.6.3 Relative Spleen Weights

With the addition of the second cohort, we updated the absolute and relative spleen weights (Figure 3). Our preliminary analysis indicates that the relative spleen weight of HEX mice and LRS mice at 4 and 8 postinjury days, individually, was significantly higher than the Sham mice at the corresponding time point. As stated in Year 02, the increased spleen weights at 4 and 8 days indicate that the addition of hemorrhagic shock induces a more severe injury than crush injury alone, including a systemic inflammatory response.

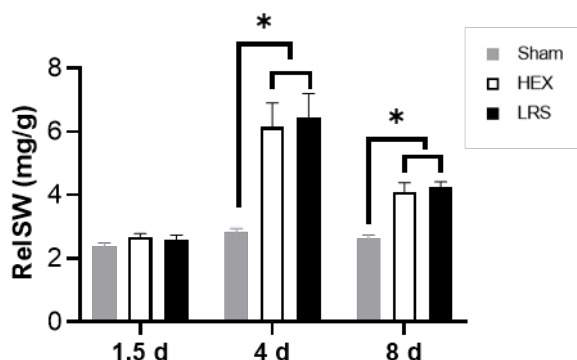


Figure 3. Relative Spleen Weight at 1.5, 4, and 8 Days Postinjury. The mean relative spleen weight (RelSW) of HEX and LRS mice at 4 and 8 postinjury days (d), individually, is significantly higher than the Sham mice at the corresponding time point (preliminary analysis). \* =  $p < .05$ . Values = mean  $\pm$  SE.  $n = 9-11$ .

In Year 02, our consultant speculated that the increased spleen weight may be due to myelopoiesis (increased production of leukocytes of the myeloid type) and encouraged us to verify the presence of myelopoiesis by harvesting the spleens and (a) performing histological (hematoxylin and eosin [H&E] staining) analysis to look for morphological changes in the spleen germinal centers, (b) performing anti-Gr-1 antibody staining, and (c) conducting flow cytometry analysis of the spleen. This consultant also recommended testing the function of spleen cells to determine whether the resuscitation fluids affect the spleen cells. In Year 03, we completed recommendations (a) and (b). However, we did not detect differences in the spleen germinal centers or observed increased anti-Gr-1 antibody staining. In addition, we examined the spleen for differences in the antigens, 7/4, CD68, CD206, CD169, CD3, and CD4. The only trend was less CD3 expression in the spleen of one HEX mouse. This observation requires follow-up.

### 3.2.1.7 Leukocyte Populations within the Plantarflexor Muscles

#### 3.2.1.7.1 Analysis of Neutrophil Populations

The flow cytometry analysis of neutrophil populations within the plantarflexor muscles was completed with the second cohort, and the summary is provided below.

**Specific Aim:** To compare the percent and number of neutrophils in crush-injured muscle between Sham and LRS or HEX mice and HEX and LRS mice.

**Methods and Measures:** Ten mice were assigned evenly to a Sham, LRS, or HEX group. The right gastrocnemius muscle of 30 mice underwent crush injury. After crush injury, the LRS or HEX mice underwent hemorrhagic shock, followed by LRS or HEX resuscitation, respectively. At 1.5 days after crush muscle injury, the right gastrocnemius and other plantarflexor muscles were harvested and assayed for the following neutrophil populations using flow cytometry:

1. Number of 7/4+ cells
2. Percent of 7/4+ cells
3. Number of 7/4Hi cells
4. Percent of 7/4Hi cells

Percent (in decimal notation) was computed as the number of positive cells divided by the number of white blood cell singlets. Number was calculated as the number of white blood cells multiplied by 100,000 times the percent of positive cells. The numbers were normalized by muscle weight (in grams).

There were no missing values for populations 1 and 2. Observations with 0.000 values were treated as missing values and were not included in the analysis. Specifically, there were three mice (1 HEX and 2 LRS mice) with missing values for populations 3 and 4. The resulting samples sizes were 9 mice for HEX and 8 for LRS for populations 3 and 4.

**Data Analysis:** To test hypotheses, we used one-sided two-sample  $t$ -tests and Wilcoxon rank sum test (Mann-Whitney U test). For the  $t$ -test, we assumed that the distributions of measurements were normal. If the tests for the equality of variances were accepted, we used the pooled method. Otherwise, we used the Satterthwaite method.

**Major Results and Conclusion:** Table 8 shows the descriptive statistics.

Table 8. Mean (Median)  $\pm$  Standard Deviation of Neutrophil Populations

Population	HEX	LRS	Sham
1	7.94 (8.35) $\pm$ 3.94	5.26 (5.83) $\pm$ 3.18	5.36 (4.90) $\pm$ 3.17
2	0.12 (0.12) $\pm$ 0.06	0.07 (0.07) $\pm$ 0.05	0.09 (0.09) $\pm$ 0.05
3	3.56 (3.01) $\pm$ 2.39	2.18 (2.13) $\pm$ 0.87	2.09 (1.50) $\pm$ 1.94
4	0.05 (0.04) $\pm$ 0.04	0.03 (0.03) $\pm$ 0.01	0.03 (0.02) $\pm$ 0.02

*Notes.* HEX = Hextend, LRS = lactated Ringer's solution. See text for details regarding each population.

Table 9 summarizes the results of the testing one-tailed hypotheses. The hypotheses are indicated in the column headings.

Table 9. Summary of Testing Neutrophil Population One-Tailed Hypotheses

Population	HEX > Sham <i>t</i> -test	HEX > Sham Nonparametric Test	LRS > Sham <i>t</i> -test	LRS > Sham Nonparametric Test
1	$p < .10$	$p < .05$	NS	NS
2	NS	NS	NS	NS
3	$p < .10$	$p < .05$	NS	NS
4	$p < .10$	$p < .10$	NS	NS

Notes. HEX = Hextend, LRS = lactated Ringer's solution, NS = not significant. See text for details regarding each population.

At the 10% significance level, all populations except the percent of 7/4+ cells were significant in the muscle of the HEX mice compared to the Sham mice. In contrast, the populations were similar between the LRS and Sham mice. At 1.5 days postinjury, the median 7/4+ cell number and median 7/4Hi cell number were statistically higher in the muscle of the HEX group (8.35, 3.01) than the Sham group (4.90, 1.50). These findings suggest that a higher number of 7/4+ cells in the muscle of the HEX group may contribute to delayed muscle recovery when compared to the Sham group. After performing this experiment, a greater sample size may be needed to detect differences between HEX and Sham groups at the 5% level.

Regarding comparisons between neutrophils in injured muscle between HEX and LRS mice, the percent of 7/4+ cells ( $p = .0358$ ) and 7/4Hi cells ( $p = .0500$ ) was higher in the HEX than in the LRS mice (Figure 4). To achieve a  $p$  value  $< .05$  for the percent of 7/4Hi cells, additional mice will need to be tested. Besides these quantitative differences, we observed a different pattern of the distribution of 7/4+ cells in the quadriceps femoris muscle (Figure 4 D–E). In this muscle of the LRS mice, these cells tended to concentrate. In contrast, in the muscle of the HEX mice, these cells tended to scatter. We will test an imaging tool to quantify this pattern.

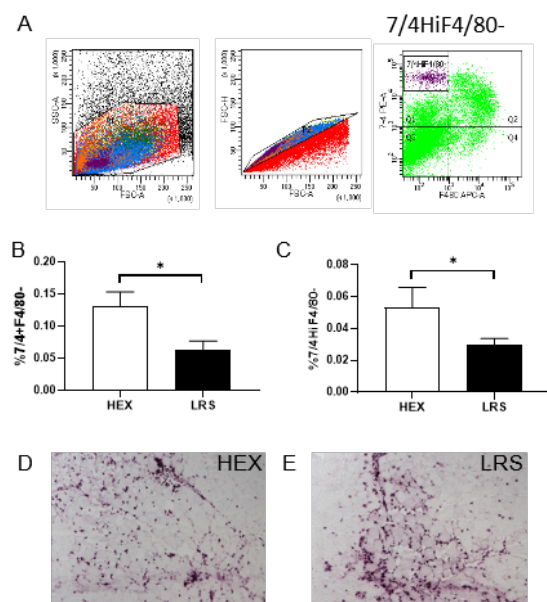


Figure 4. Neutrophils in Crush-Injured Muscle at 1.5 Days Postinjury. A) Flow cytometry gating strategy used to identify neutrophils (7/4+F4/80-) in injured plantarflexor muscles. Total leukocytes were gated within the leukocyte gate using FSC-Area vs. SSC-Area. Singlets were gated using FSC-Height vs. FSC-Area. 7/4+F4/80- cells were gated on the dot plot. B–C) Proportion of 7/4+ and 7/4Hi neutrophils in the injured PF muscles.  $n = 8-10$ ; B) \*LRS vs. HEX,  $p < .05$  and C) \*LRS vs. HEX,  $p = .05$ . D–E) 7/4 immunolabeling in injured quadriceps femoris muscle. The pattern of 7/4+ cells in the muscle of HEX mice is scattered (D), whereas in the muscle of LRS mice, the cells are concentrated (E). Bar = 100  $\mu$ m.

### 3.2.1.7.2 Analysis of Macrophage Populations

The analysis of macrophage populations as detected with flow cytometry within the plantarflexor muscles was completed, and the summary is provided below.

**Specific Aims:** To compare the percent and number of M1 and M2 macrophages in crush-injured muscle between Sham, HEX, and LRS mice.

**Methods and Measures:** Mice were assigned to nine groups (three treatments  $\times$  three time points). The three treatments were Sham, HEX, and LRS, and the three time points were 1.5, 4, and 8 days (after crush muscle injury). The right gastrocnemius muscle of 91 mice underwent crush injury. After crush injury, the LRS or HEX mice underwent hemorrhagic shock, followed by LRS or HEX resuscitation, respectively. At 1.5, 4, or 8 days after crush muscle injury, the mice underwent euthanasia, and the right gastrocnemius muscle was harvested and assayed for the following macrophage populations using flow cytometry. For this analysis, group sizes ranged from 9–11, totaling 89 mice.

The six macrophage populations included the following:

1. CD68+MHCII+ (both CD68 and MHCII antigens are present),
2. CD11b+F4/80+CD68+MHCII+ (four antigens of CD11b, F4/80, CD68, and MHCII are present),
3. Arg-1+CD68+ (both Arg-1 and CD68 antigens are present),
4. CD206+CD68+F4/80+Arg-1+ (both CD206 and CD68 antigens are present),
5. CD206+CD68+F4/80+Arg-1+ (four antigens of CD206 CD68, F4/80, and Arg1 are present), and
6. 7/4–F4/80+CD68+ (F4/80 and CD68 antigens are present, but the 7/4 antigen is absent).

For each population, percent and number of positive cells were measured and analyzed. Percent (in decimal notation) was computed as the number of positive cells divided by the cell number of WBC singlets. Number was calculated as the number of WBCs multiplied by 100,000 times the percent of positive cells. The numbers were normalized by muscle weight (in grams).

**Data Analysis:** Under the assumptions of (1) normally distributed data and (2) variance homogeneity, we tested for the effects of the treatment and time using two-way ANOVA. Because ANOVA is robust to unequal variances when group sample sizes are similar, we used ANOVA in all cases where the model fit the data adequately—specifically for populations 1, 2, and 4—even if posthoc tests pointed to unequal variances. For each significant factor with 3 levels, we then performed multiple comparisons using *t*-tests and Bonferroni adjustments. If the folded *F*-test for equality of variances was significant, we performed a Satterthwaite *t*-test (suitable for unequal variances). If the folded *F*-test was not significant, we performed a pooled *t*-test (suitable for equal variances). In addition, we used Kruskal-Wallis tests to compare three treatments (time points) within each time point (treatment) for populations 3 and 5–8 because the ANOVA model did not fit the data adequately. Because none of the Kruskal-Wallis tests were significant, we did not perform any posthoc testing. However, if a test were significant, we would perform multiple comparisons via Wilcoxon rank sum test (Mann-Whitney U test) with Bonferroni adjustments. Each claim was demonstrated at the 5% level.

**Major Results and Conclusion:** The only macrophage population that differed among the treatment groups was population 2, M1 macrophages, CD11b+F4/80+CD68+MHCII+ (Table 10, Figure 5). Unlike the Sham and HEX groups, the LRS group maintained a consistent percent of M1 macrophages at 1.5 and 4 days postinjury. At 8 days postinjury, the mean percent of M1 macrophages was statistically higher in the muscle of HEX mice than LRS mice. Furthermore, the morphology of these cells also indicated differences. The discrete, completely filled morphology observed in the muscle of the LRS mice is similar to what can be observed in macrophages present in uninjured muscle or areas. In contrast, the clustered and ring-like morphology is more similar to that observed in injured areas. Collectively, the numerical and morphological findings suggest LRS treatment limits the M1 macrophage activity greater than HEX treatment, which may promote a faster recovery than HEX treatment.

Table 10. Mean (Median)  $\pm$  Standard Deviation for Percent<sup>a</sup> of CD11b+F4/80+CD68+MHCII+ (Macrophage) Populations

	1.5 Days	4 Days	8 Days
Sham	0.034 (0.033) $\pm$ 0.01	0.090 (0.087) $\pm$ 0.03	0.089 (0.087) $\pm$ 0.03
HEX	0.031 (0.025) $\pm$ 0.02	0.078 (0.063) $\pm$ 0.05	0.089 (0.073) $\pm$ 0.04
LRS	0.032 (0.031) $\pm$ 0.02	0.059 (0.046) $\pm$ 0.03	0.029 (0.029) $\pm$ 0.02

Notes. HEX = Hextend, LRS = lactated Ringer's solution.

<sup>a</sup>Percent in decimal notation.

8 days, HEX versus LRS,  $p = .0062$ ; 8 days, Sham versus LRS,  $p < .0001$ .

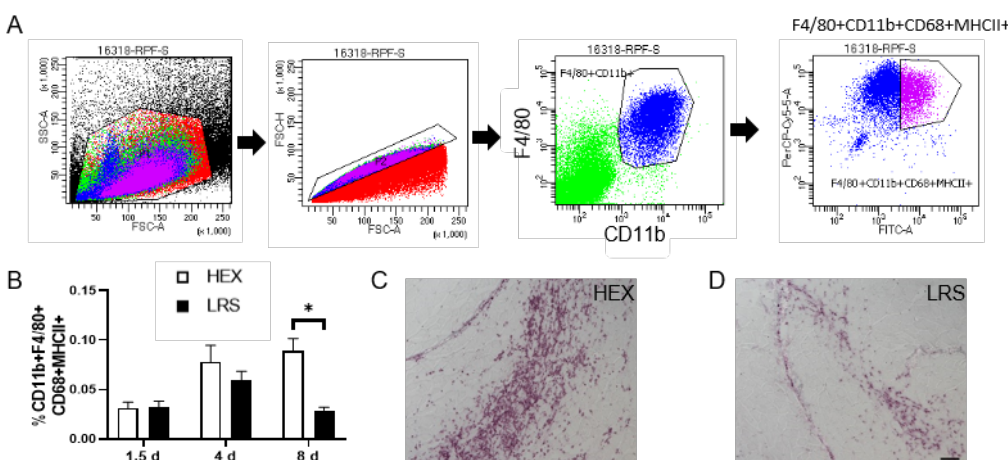


Figure 5. M1 Macrophages in Crush-Injured Muscle at 1.5, 4, and 8 Days (d) Postinjury. A) Flow cytometry gating strategy used to identify M1 macrophages (CD11b+F4/80+MHCII+CD68+ cells) in crush-injured plantarflexor muscle. B) Proportion of M1 macrophages.  $n = 9-10$ .  $*p < .05$ . C) Immunolabeling of CD68+ cells in a crush-injured quadriceps femoris muscle at 8 d postinjury. CD68+ cells in a HEX mouse are clustered and appears as ring-like structures, whereas these cells in an LRS mouse are more discrete and completely filled. Bar = 100  $\mu$ m

### 3.2.1.8 Leukocyte Populations Within the Quadriceps Femoris Muscle

Ten-micron-thick cross-sections were cut from the quadriceps femoris muscles and underwent H&E staining to examine the general morphology of the muscles and underwent immunolabeling to detect specific leukocyte populations.

For mice euthanized at 1.5 days postinjury, we detected eosin-stained pale fibers, indicating the presence of injured fibers from the crush stimulus. The features of these fibers (e.g., presence of internal nuclei) appeared similar among the Sham, HEX, and LRS groups. We counted the number of fibers and quantified the cross-sectional area per fiber. In many cases, these fibers appeared to merge, so the more suitable approach was to quantify the total pale fiber cross-sectional area (TPFC). Our preliminary analysis is that the TPFC was approximately two-fold higher in the muscle of Sham mice compared to HEX and LRS mice. However, the TPFC varied among these groups, depending on the specific quadriceps femoris muscle. In the vastus lateralis (VL), the TPFC was highest in the Sham mice compared to the two shock groups, whereas in the rectus femoris, the TPFC was lowest in the HEX mice compared to the Sham mice. In the vastus medialis (VM), the TPFC was highest in the HEX group compared to Sham and LRS groups. Additional work needs to be completed to confirm these findings.

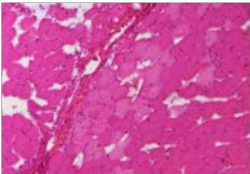
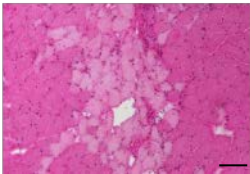
		Total Pale Fiber Cross-Sectional Area				
		All	VL	VM	RF	
Sham		Sham	.455	.204	.063	.084
	HEX	HEX	.173	.058	.119	.006
		LRS	.213	.099	.031	Not done
LRS		Note: All = Area combined for vastus lateralis (VL), vastus medialis (VM), and rectus femoris (RF). Values = median in mm <sup>2</sup> . Not done = insufficient number of muscles with pale fibers. Group sizes (excluding LRS-RF) 8–10.				

Figure 6. Crush-injured Quadriceps Femoris Muscle. At 1.5 days postinjury, the injured fibers appear as pale eosin-stained fibers in the Sham, HEX, and LRS groups. Bar = 100  $\mu$ m. Our preliminary analysis is that TPFC is approximately twofold higher in the muscle of the Sham mice compared to the HEX and LRS mice. However, the TPFC varies among these groups depending on the specific quadriceps femoris muscle.

As proposed, we have immunolabeled the sections for CD68-positive, CD206-positive, and F4/80-positive cells. To compare with the flow data, we added immunolabeling for CD11b- and 7/4-positive cells. We completed the immunolabeling and captured images to perform computer-assisted analysis. Analysis is pending.

### 3.2.1.9 Myofiber Regeneration in the Quadriceps Femoris Muscle

To examine myofiber regeneration, 10-micron-thick cross-sections were cut from the quadriceps femoris muscles harvested from 96 and 192 hours postinjury. Sections underwent H&E staining to examine the general morphology of the muscles (Figure 7) and underwent immunolabeling to detect myofibers positive for developmental myosin heavy chain (dMHC; Figure 8).

Figure 7 shows that myofiber regeneration was evident at 4 and 8 days postinjury. At 4 days, nuclei accumulated and early-stage regenerating myofibers appeared in Sham, HEX, and LRS mice (Figure 7 A, C, and E). At 8 days, the nuclear accumulation diminished and later-stage regenerating myofibers were more commonly observed in the Sham and LRS mice. The characteristics of early-stage myofiber regeneration remained evident in the HEX mice.

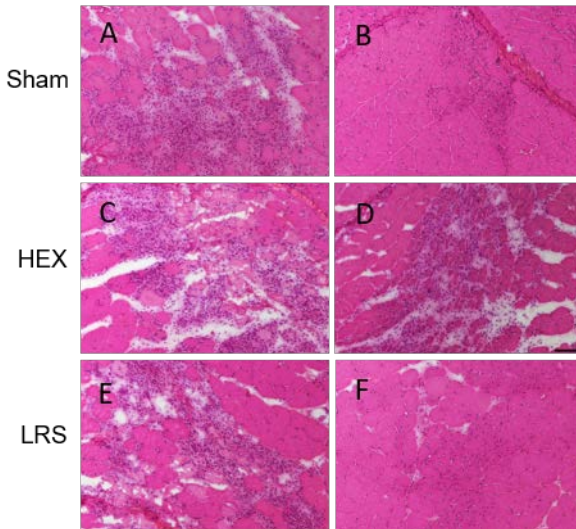


Figure 7. Crush-injured Quadriceps Femoris Muscle Cross-section Images at 4 and 8 Days (d) Postinjury. At 4 d, early-stage myofiber regeneration is evident by an accumulation of nuclei (purple objects) and presence of early-stage or few regenerating myofibers in all three groups. At 8 d, the nuclear accumulation has diminished, myofibers are arranged compactly, and later-stage regenerating muscle fibers are more commonly observed only in the Sham and LRS groups. The characteristics of early-stage myofiber regeneration remain evident in the HEX mice. Bar = 100  $\mu$ m.

Figure 8 shows that dMHC expression present in regenerating myofibers was intense at 4 days postinjury in all three groups (A, C, and E). At 8 days, larger dMHC+ fibers were present, but the intensity varied (B, D, and E). In the Sham and LRS groups, the intensity of both smaller and larger fibers was similar. However, in the HEX group, the larger fibers were less intense than those in the two other groups. Furthermore, smaller, less intense fibers were present in the HEX group. These data suggest delayed or impaired dMHC expression in the HEX group. An analysis is underway to quantify these differences.

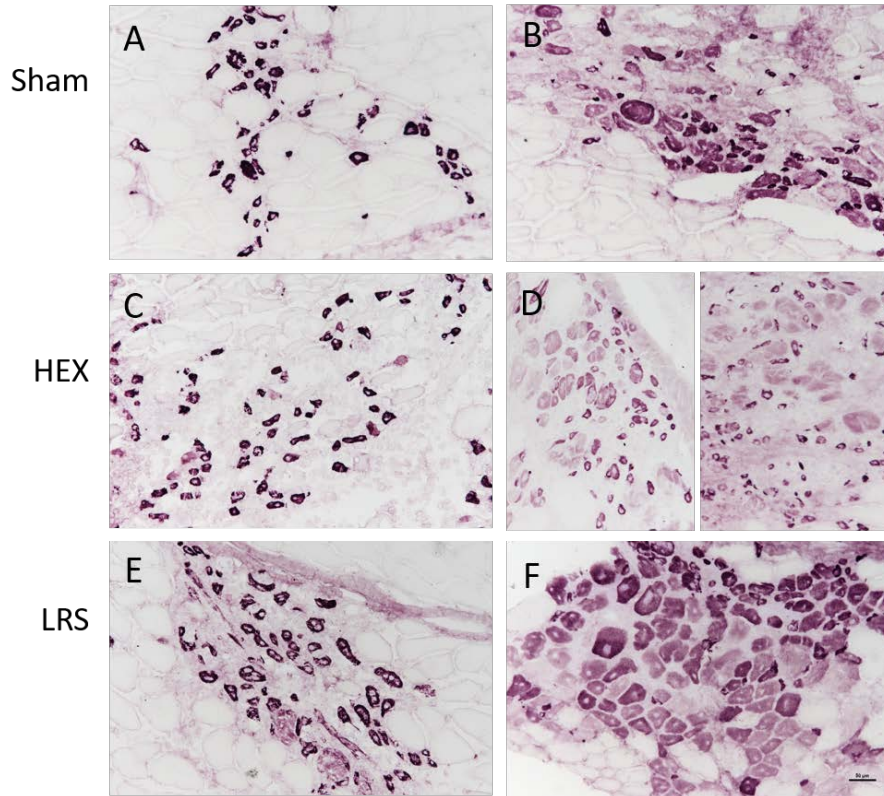


Figure 8. Developmental Myosin Heavy Chain (dMHC) Expression in Crush-injured Quadriceps Femoris Muscle Cross-section Images at 4 and 8 Days (d) Postinjury. A, C, and E) At 4 days, dMHC expression present in regenerating myofibers is intense. B, D, and F) At 8 days, larger dMHC+ fibers are present, but the intensity varies. In the Sham (B) and LRS (F) groups, the intensity of both smaller and larger fibers is similar. However, in the HEX group, the larger fibers are less intense (D) than those in the two other groups. Furthermore, smaller, less intense fibers are present in the HEX group. Bar = 50  $\mu$ m.

### 3.2.1.10 Plantarflexor Muscle Function

We completed data collection and analysis for plantarflexor muscle function with cohorts 1 and 2. A summary is provided below.

**Specific Aims and Hypotheses:** Three specific aims were analyzed. Two aims related to measures of muscle torque percent change and differences. A third aim focused on time to fatigue.

Hypothesis 1 – The muscle function (torque) of HEX and LRS mice is decreased relative to that of Sham mice at any postinjury time point.

Hypothesis 2 – The muscle function (torque) of HEX mice is decreased relative to that of Sham and LRS mice.

Hypothesis 3 – The time to fatigue is shorter in the HEX mice than the Sham or LRS mice at 8 days postinjury.

**Methods:** The same mouse procedures were used for macrophage analysis. Data from 84 mice were usable (Table 11).

Table 11. Plantarflexor Muscle Function Group Sizes

	1.5 d	4 d	8 d
Sham	9	10	10
HEX	10	9	9
LRS	9	9	9

*Note.* HEX = Hextend, LRS = lactated Ringer's solution.

The function of the plantarflexor (calf) muscles was evaluated using generated maximum isometric torque once immediately before one set of 50 eccentric contractions (1st\_IsoT) and twice immediately after the 50 eccentric contractions (2nd\_IsoT and 3rd\_IsoT). The following measurements were collected: mouse body weight at euthanasia (kilograms, BWEU), right plantarflexor muscle weight (grams, RPF), three maximum isometric torque values (as mentioned above, 1st\_IsoT, 2nd\_IsoT, and 3rd\_IsoT), two maximum eccentric torque values (mN-m) of the 1st eccentric contraction (EC0) and the 50th eccentric contraction (EC49). One value for 1st\_IsoT for LRS at 4 days was missing.

**Measures:** Because body weight and muscle weight can affect muscle function, and a larger mouse or a bigger muscle can generate more muscle torque, it is common to normalize muscle torque by body weight or muscle weight. Therefore, the isometric and eccentric torque values were divided by body weight or muscle weight:

- a. 1st\_IsoT/BWEU, 2nd\_IsoT/BWEU, 3rd\_IsoT/BWEU, EC0/BWEU, and EC49/BWEU
- b. 1st\_IsoT/RPF, 2nd\_IsoT/RPF, 3rd\_IsoT/RPF, EC0/RPF, and EC49/RPF

The differences were defined as the “later” normalized measure minus the “previous” normalized measure (e.g., 2nd\_IsoT /BWEU – 1st\_IsoT/BWEU, 3rd\_IsoT /BWEU – 1st\_IsoT/BWEU and EC49/BWEU – EC0/BWEU). The percent change was defined as

$$\frac{\frac{y}{w} - \frac{x}{w}}{\frac{x}{w}} = \frac{y - x}{x} = \frac{y}{x} - 1$$

where  $x, w > 0$ ,  $w$  denotes normalization using BWEU or RPF values. The ratio on the left-hand side does not depend on  $w$ ; thus, both normalizations reduce to the original non-normalized measures.

In addition, time to fatigue (in seconds), defined as the time until the eccentric torque reduced to 50% percent of the 1st eccentric torque, was recorded.

**Data Analysis:** To demonstrate Aim 1, we compared combined HEX and LRS relative to Sham. To demonstrate Aims 2 and 3, we compared HEX versus Sham and HEX versus LRS. We used the nonparametric one-tailed exact test and the Wilcoxon rank sum test. We set the alpha at the 10% significance level because of the investigative nature of this study. Specifically, this plantarflexor muscle function testing has not been performed previously using data from HEX and LRS treatments. In addition, all studied groups had small sizes. We also used  $t$ -tests, but the

validity of assumptions was not confirmed. Therefore, only the statistically significant results from the nonparametric tests are reported.

**Major Results:** Tables 12–13 present the statistically significant results (alpha level set at .10). For Aim 1 (Table 5), the reduction in isometric torque (before and immediately after one set of eccentric contractions) was greater in the HEX/LRS versus Sham at 1.5 days. Also, the reduction of eccentric torque was greater in the HEX/LRS versus Sham at 1.5 days. At 8 days, the reduction in isometric torque (before and 2–3 minutes after one set of eccentric contractions) is greater in the Sham versus the HEX/LRS mice. There were no statistically significant results comparisons at 4 days postinjury.

Table 12. Aim 1 - Significant Medians of Plantarflexor Muscle Torque - Sham Group Versus HEX and LRS Groups Combined

Days	Variables	Median	<i>p</i>
1.5	(2nd_IsoT - 1st_IsoT)/1st_IsoT	-0.57 vs. -0.69	.05
	(EC49 - EC0)/EC0	-0.79 vs. -0.82	.02
8	(3nd_IsoT - 1st_IsoT)/1st_IsoT	-0.44 vs. -0.37	.04
	(3nd_IsoT - 1st_IsoT)/BWEU	-60.04 vs. -50.38	.08
8	(3nd_IsoT - 1st_IsoT)/RPF	-8.46 vs. -6.39	.05

*Notes.* The first value in column 2 is the Sham group value. The second value in column 2 is the value of HEX and LRS groups combined.

Alpha set at .10. vs = versus. One-tailed *p* values of nonparametric tests.

For Aim 2, Table 13 shows the significant Sham and HEX group comparisons. At 1.5 days, the reduction in eccentric torque was greater in the HEX versus the Sham group. At 8 days, the reduction in isometric torque was greater in the Sham versus the HEX group.

Table 13. Aim 2. Significant Medians of Plantarflexor Muscle Torque Between Sham and HEX Groups

Days	Variables	Median	<i>p</i>
1.5	(EC49 - EC0)/EC0	-0.76 vs. -0.78	.03
8	(2nd_IsoT - 1st_IsoT)/1st_IsoT	-0.64 vs. -0.54	.07
8	(3nd_IsoT - 1st_IsoT)/1st_IsoT	-0.44 vs. -0.38	.05
8	(3nd_IsoT - 1st_IsoT)/BWEU	-60.04 vs. -44.37	.08
8	(3nd_IsoT - 1st_IsoT)/RPF	-8.46 vs. -5.71	.08

*Notes.* The first value in column 2 is the Sham group value. The second value in column 2 is the value of HEX group.

Alpha set at .10. vs = versus. One-tailed *p* values of nonparametric tests.

For the LRS and HEX group comparisons, at 8 days, the reduction in isometric and eccentric torque was greater in the LRS versus the HEX group: (2nd\_IsoT - 1st\_IsoT)/1st\_IsoT, -0.59 vs. -0.54, *p* = .0951 (one-tailed) and (EC49 - EC0)/EC0, -29.77 vs. -19.54, *p* = .0680 (one-tailed). There were no statistically significant results for HEX and LRS group comparisons at 1.5 and 4 days.

Regarding Aim 3, while there was a statistically significant result (*p* = .0975) comparing the time-to-fatigue between HEX and LRS (Median = 18 and 20, respectively), the corresponding

standard deviation values 4.9 and 23.2 suggest that the LRS group median is not an adequate representation of the data. Therefore, this comparison is not considered to be valid.

**Conclusions:** The two of the three hypotheses were partially supported. At 1.5 days, the reduction in muscle function is greater in the HEX/LRS or HEX versus the Sham mice. However, at 8 days, the reduction in muscle function is greater in the Sham versus HEX/LRS and HEX mice. Also, at 8 days, the reduction in muscle function is greater in the LRS versus HEX mice. The possibility exists that more normal muscle fibers may be present in the Sham and LRS mice than the HEX mice (Figures 7 and 8) to generate isometric torque at the start and then fatigue later, which would result in a greater reduction in isometric torque after eccentric contractions. In future studies, the group sizes may need to be larger and/or the eccentric contraction protocol may need to be more intense to detect more robust differences.

3.2.2 Subtask 6: Review Hypothesis 1 and 2 results.

We continued to review the results of Hypothesis 2B.

3.2.3 Subtask 7: Write and/or review report.

We were delayed in starting this report.

3.2.4 Subtask 8: Disseminate results.

See Section 6.0 Products.

3.2.5 Subtask 9: Submit and receive animal protocol amendment approval.

We submitted and received animal protocol amendment approval for eight amendments. See Section 5.0 for details.

3.2.6 Specific Aims 2 and 3: Major Task 1: Hypothesis 3 Testing – C57BL/6 male mice 1–190

3.2.6.1 Subtask 1: Complete animal procedures.

3.2.6.1.1 Hypothesis 3 Animal Procedures

As of 19-Jul-2019, for Hypothesis 3 (H3), we have used 275 out of 533 mice (52%). Eleven of these mice experienced an early death, and five of these mice served as controls for RT-PCR assays. Therefore, we completed this subtask, and a total of 259 of mice assigned to the six different groups were euthanized at their assigned euthanasia date. Table 14 provides additional details.

Table 14. H3 Mouse Number and Percent<sup>a</sup>

Event	Number	Percent
Start total	270	NA
Tested, but did not undergo trauma	91 of 92	99
Tested and underwent crush injury	178	NA
Sham completion <sup>b</sup> and survival <sup>c</sup>	86 of 86	100
Shock completion <sup>b</sup>	89 of 92	97
Shock survival <sup>d</sup>	82 of 89	92

Notes. NA = not applicable. H3 = Hypothesis 3

<sup>a</sup>These values were determined from mice that had a euthanasia time point between 17-December 2018 and 19-July-2019.

<sup>b</sup>Completion = The mouse underwent crush injury followed by sham shock or shock/fluid resuscitation and recovered from anesthesia.

<sup>c</sup>Survival = The mouse was euthanized at its assigned time point.

### 3.2.6.1.2 Hematological Parameters

Since we had access to a Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter), we measured these hematological parameters—RBC count, hemoglobin, and mean corpuscular volume to calculate hematocrit—from whole blood obtained at the time euthanasia. The circulating RBC count and hemoglobin level of the Shock mice were lower at 1.7 and 4 days than the count of the Sham mice, but these parameters did not appear to be different between the Shock mice exposed to normobaric normoxia (NB-shock) and hypobaric hypoxia (HB-shock, Figures 9 and 10, preliminary analysis). The hematocrit results followed the same pattern (data not shown).

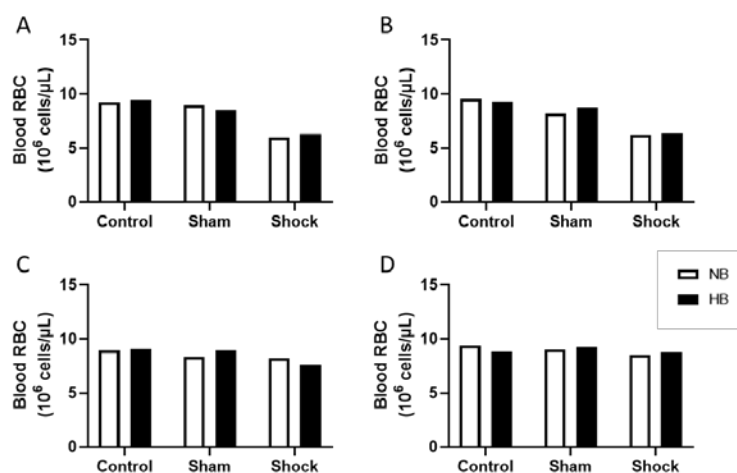


Figure 9. Red Blood Cell (RBC) Count in Whole Blood. The mean RBC count of the Shock mice is lower at 1.7 and 4 days than the count of the Sham mice. Similar counts occur between NB-shock and HB-shock at all time points. A) 1.7 days postinjury,  $n = 7-9$ ; B) 4 days postinjury,  $n = 12-14$ , C) 8 days postinjury,  $n = 11-14$ , and D) 14 days postinjury,  $n = 11-14$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. No error bars are included because group sizes are not final.

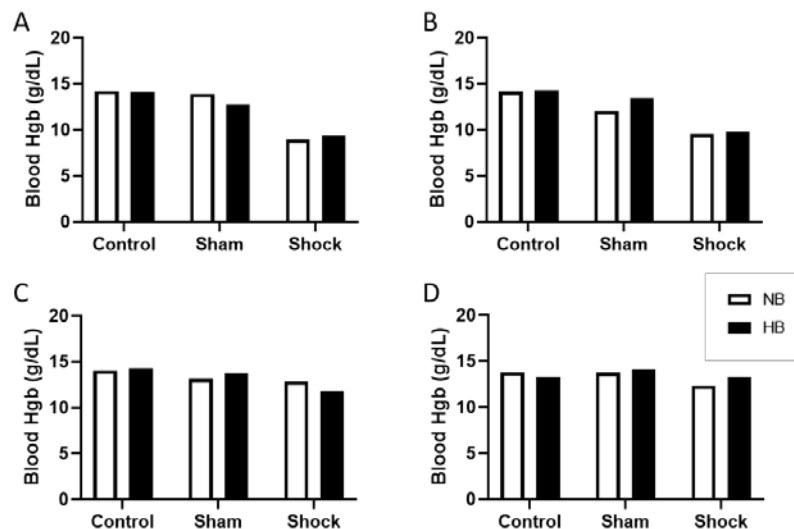


Figure 10. Hemoglobin (Hgb) Level in Whole Blood. The mean Hgb level of the Shock mice is significantly lower at 1.7 and 4 days than the level of the Sham mice. Similar counts occur between NB-shock and HB-shock at all time points. A) 1.7 days postinjury,  $n = 7-9$ ; B) 4 days postinjury,  $n = 12-14$ , C) 8 days postinjury,  $n = 11-14$ , and D) 14 days postinjury,  $n = 11-14$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. No error bars are included because group sizes are not final.

3.2.6.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (including data entry) for 190 mice.

### 3.2.6.2.1 Flow Cytometry

Flow cytometry assays involve detecting the number and percentage of blood leukocytes, spleen leukocytes and reticulocytes, and muscle leukocytes.

#### 3.2.6.2.1.1 Muscle Leukocytes

To date, we have collected muscle leukocytes from 123 right plantarflexor muscles and 125 left plantarflexor muscles from 125 mice. (Two right plantarflexor muscles were inadvertently processed for immunohistochemistry instead of flow cytometry.) Therefore, we have collected 51% and 52% of the proposed muscles. The two muscle leukocyte flow panels for this analysis are as follows:

- CD11b, F4/80, 7/4, Ly-6C and Ly-6G for neutrophils, monocytes, and macrophages; and
- CD11b, CD68, CD206, Arg-1, and MHCII for M1 and M2 macrophages.

These analyses will be performed in Year 04.

#### 3.2.6.2.1.2 Blood Leukocytes

Before blood undergoes flow cytometry, whole blood samples after RBC lysis buffer treatment (to remove RBCs) are processed by the Z2 Particle Counter (Beckman Coulter, Indianapolis, IN) with a cell diameter size range setting at 5.5–10 microns. This process provides the number of blood leukocytes per mouse. Because we will have blood available for up to 480 mice, and H2B data indicate that a group size of 10 may be insufficient for Shock mice, we plan to assay up to 15 blood samples per group for the Sham and Shock mice,  $N = 320$ ). Therefore, the status of this analysis is as follows: control mice, 85%; Sham mice, 52–55%; and Shock mice, 52–57%.

Figure 11 shows the mean number of blood leukocytes per group. Since the Sham and Shock groups are approximately only 50% complete, statistical analysis is premature.

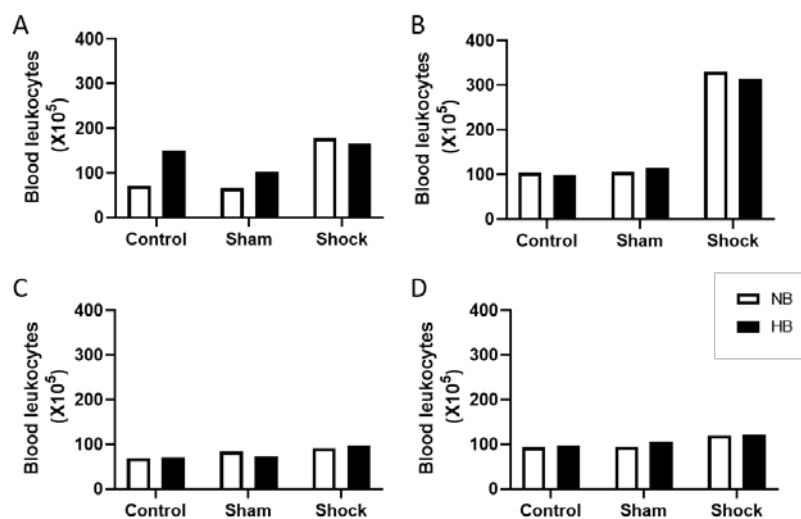


Figure 11. Blood Leukocyte Number. Similar counts occur between NB-shock and HB-shock at all time points. A) 1.7 days postinjury,  $n = 6-9$ ; B) 4 days postinjury,  $n = 8-14$ , C) 8 days postinjury,  $n = 8-14$ , and D) 14 days postinjury,  $n = 10-14$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. No error bars are included because group sizes are not final.

To date, blood from 226 mice has undergone flow cytometry. The blood leukocyte flow panels are directed toward quantifying the number and percentage of neutrophils and monocytes. The two neutrophil panels are Ly6G+Ly6C+CD11b+ and F480-7/4HiCD11b+, and the one monocyte panel is Ly6C+Ly6G-CD11b+. As these panels have only been completed for 61% of mice, statistical analysis is premature.

In addition, we observed another blood leukocyte subpopulation increase in the shock mice, F4/80+7/4-, at 4 days postinjury. Thus far, preliminary statistical analysis indicates a significant mean difference between the NB-shock and NB-sham group,  $11.70 \pm 6.39$  (SE) vs.  $4.90 \pm 2.20$  (SE). These cells could be eosinophils.

### 3.2.6.2.1.3 Spleen Leukocytes

Upon the advice of our consultant, in H3, we followed up regarding the splenomegaly observed in the shock mice in four ways. We investigated relative spleen weight, spleen wet-to-dry weights, overall leukocyte number, and reticulocyte phenotypes.

#### 3.2.6.2.1.3.1 Relative Spleen Weight

Similar to H2B, we observed an increase in relative spleen weight in the shock mice at 4 and 8 days postinjury in comparison with 1.7 days (Figure 12). Additional mice are needed to determine whether differences exist between NB-shock and HB-shock mice (Figure 12).

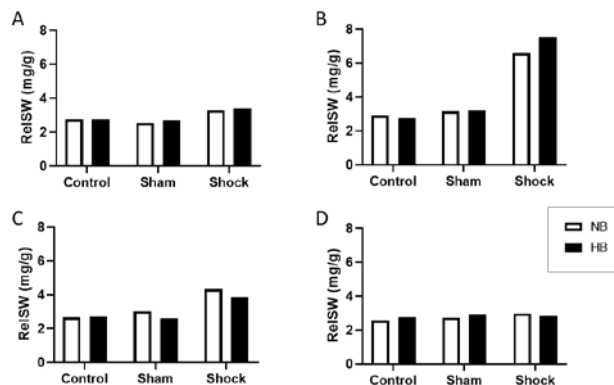


Figure 12. Relative Spleen Weight. The mean relative spleen weight (absolute spleen weight divided by body weight at time of euthanasia) was higher at 4 and 8 days in comparison with 1.7 days and 14 days. Similar values are observed between the NB-shock and HB-shock at any time point. A) 1.7 days postinjury,  $n = 4-6$ ; B) 4 days postinjury,  $n = 6-9$ , C) 8 days postinjury,  $n = 9-11$ , and D) 14 days postinjury,  $n = 9-12$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. Values = mean. No error bars are included because group sizes are not final.

### 3.2.6.2.1.3.2 Spleen Wet-to-Dry Weight Ratio

The consultant suggested we rule out whether the increased spleen wet was due to edema, so we measured the wet and dry weights of 42 spleens from different postinjury time points and treatment groups, ranging from 2–3 spleens per group. After obtaining a wet weight, the dry weight was determined by placing the spleen in an incubator at  $-60^{\circ}\text{C}$  for 4 days. Then, a wet-to-dry weight ratio was calculated. If significant edema were present in the spleen of shock mice, then we would expect the mean wet-to-dry weight ratio to be at least twofold higher (based on relative spleen weight values) in these mice compared to the other mice. The overall wet-to-dry ratio range was 3.38–5.31 for 40 mice and 2.55 and 2.86 for two mice. Since the mean wet-to-dry ratio of the NB-control mice (at all postinjury time points) was 4.36, NB-sham mice was 4.79 (4 days postinjury), and NB-shock mice was 4.52 (4 days postinjury), we concluded that the increased spleen weight observed in the shock mice (at 4 days postinjury) is not strongly related to edema.

### 3.2.6.2.1.3.3 Overall Spleen Leukocyte Number

Figure 13 shows the overall spleen leukocyte number per group. (Note: These values are not normalized to spleen absolute weight because leukocytes are the primary cells of the spleen. A change in spleen weight will be proportional to a change in leukocyte number.) Our preliminary statistical analysis indicates that the spleen leukocyte number of the Shock mice significantly differed from the corresponding Sham mice at 4 days postinjury. For the NB-shock mice, the number of leukocytes was 3-fold higher compared to the NB-sham mice, whereas the number of leukocytes increased 2.4-fold in the HB-shock mice versus HB-sham. However, this analysis did not yield indicate a significant difference between the two Shock groups, although the group size is small. Regarding 8 days postinjury, while no significant difference was detected between the NB-sham and NB-shock groups, a significant difference was detected between the HB-sham and HB-shock groups exposed to hypobaric hypoxia. The leukocyte number of the HB-shock group was 2-fold higher than that of the HB-sham group.

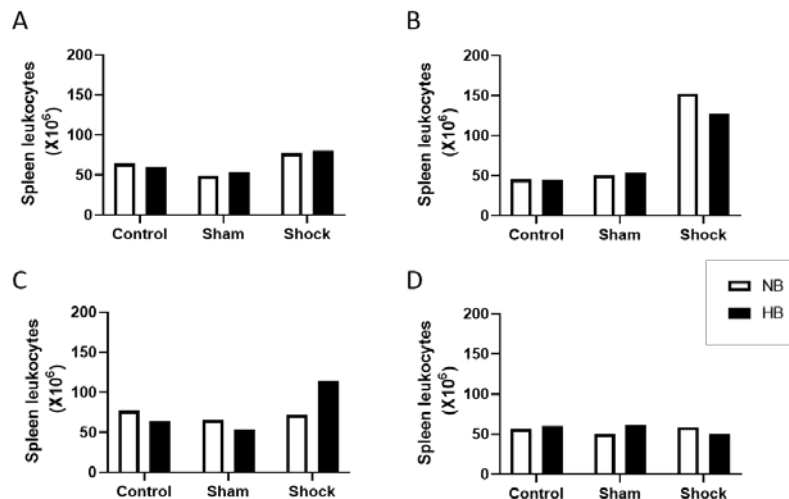


Figure 13. Spleen Leukocyte Number. Mean spleen leukocyte number of the Shock mice significantly differs from that of the corresponding Sham mice at 4 days postinjury (preliminary analysis). Similar values are observed between the NB-shock and HB-shock at any time point, but group sizes are small. A) 1.7 days postinjury,  $n = 3-4$ ; B) 4 days postinjury,  $n = 5-6$ , C) 8 days postinjury,  $n = 5-7$ , and D) 14 days postinjury,  $n = 5-8$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. Values = mean. No error bars are included because group sizes are not final.

#### 3.2.6.2.1.3.4 Spleen Reticulocytes

Because the spleen can serve as an extramedullary site of erythropoiesis, we have investigated whether the splenomegaly is related to erythropoiesis in the spleen. As part of H3, we have performed flow cytometry to detect immature (CD71+Ter119+) and mature (CD71-Ter119+) reticulocytes. Figures 14–16 show the gating strategy and results for immature and mature reticulocyte populations. Based upon a total of 129 spleens, a significant mean difference occurred in immature reticulocytes between the Shock mice and corresponding Sham mice. These data indicate that the spleen serves as an extramedullary site of erythropoiesis in this model. In addition, the number of immature reticulocytes was significantly lower (41%) in the HB-shock mice compared to the NB-shock mice. More recently, the consultant advised us to determine whether the immature reticulocytes are proliferating in the NB-shock mice at 4 days postinjury and undergoing apoptosis in the HB-shock mice to rule out whether simulated flight may impair or delay extramedullary site of erythropoiesis.

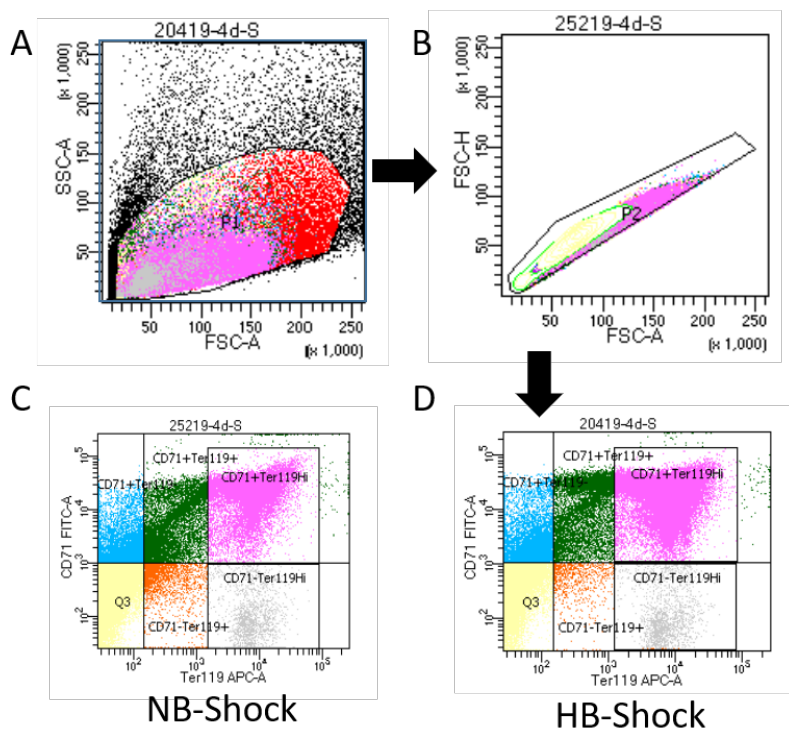


Figure 14. Spleen Reticulocytes Gating Strategy. A) Dot plot of overall population of spleen cells (P1). B) Dot plot of singlets of spleen cells (P2). C and D) Dot plots of immature reticulocytes (upper right quadrant) and mature reticulocytes (lower right quadrant) from one NB-shock mouse (C) and one HB-shock mouse (D) at 4 days postinjury. NB-shock = normobaric normoxia exposure after hemorrhagic shock; HB-shock = hypobaric hypoxia exposure after hemorrhagic shock.

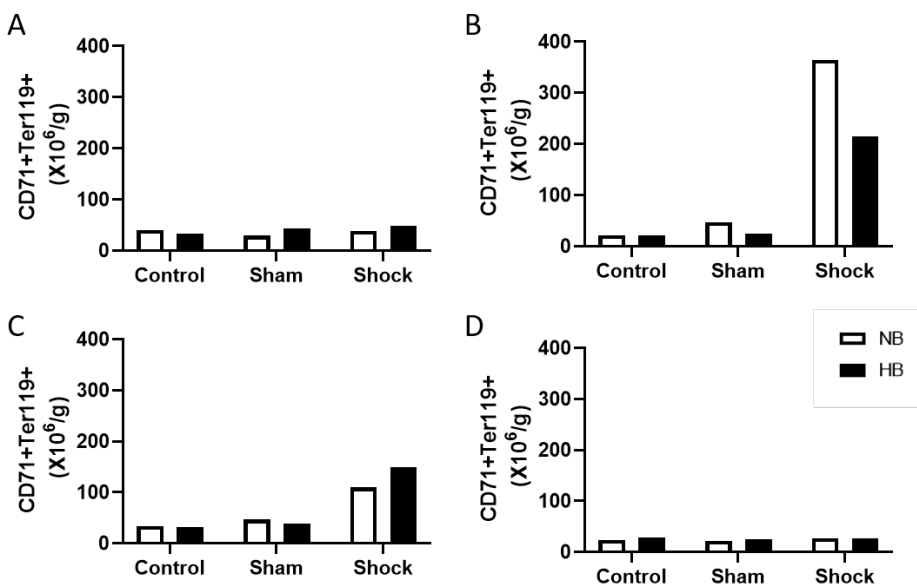


Figure 15. Relative Spleen Immature Reticulocyte Number. The mean number of immature reticulocytes in the spleen of NB-shock and HB-shock is 7.6-fold and 8.5-fold higher compared to the corresponding Sham mice at 4 days postinjury. In addition, the NB-shock is 1.7-fold higher compared to HB-shock at 4 days postinjury. Values are normalized by absolute spleen weight (g). A) 1.7 days postinjury,  $n = 2-4$ ; B) 4 days postinjury,  $n = 5-6$ , C) 8 days

postinjury,  $n = 5-7$ , and D) 14 days postinjury,  $n = 5-8$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. Values = mean. No error bars are included because group sizes are not final.

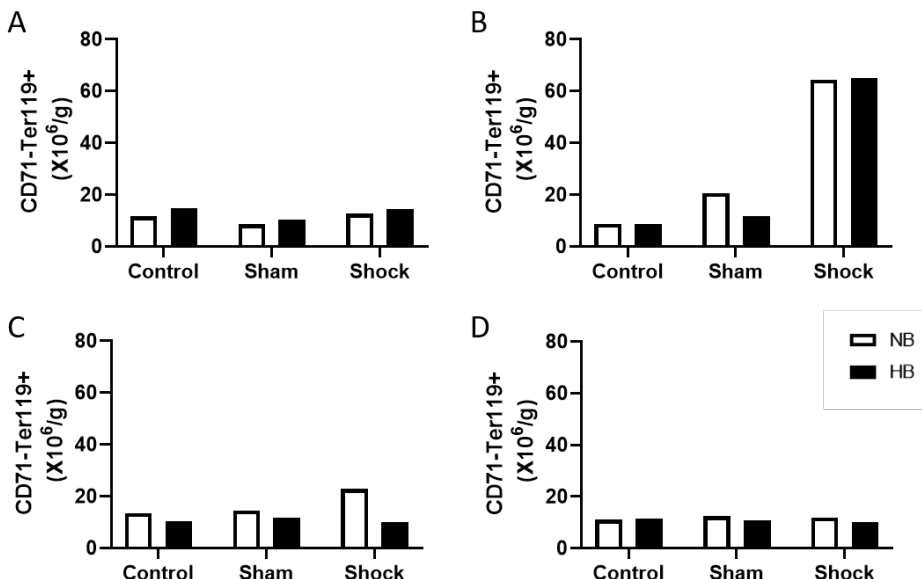


Figure 16. Relative Spleen Mature Reticulocyte Number. The mean number of mature reticulocytes in the spleen of NB-shock and HB-shock is similar at 4 days postinjury. Values are normalized by absolute spleen weight. A) 1.7 days postinjury,  $n = 2-4$ ; B) 4 days postinjury,  $n = 5-6$ , C) 8 days postinjury,  $n = 5-7$ , and D) 14 days postinjury,  $n = 6-8$ . □ = normobaric normoxia exposure; ■ = hypobaric hypoxia exposure. Values = mean. No error bars are included because group sizes are not final.

In addition, the consultant advised us to examine whether spleen neutrophils or monocytes are ingesting these immature reticulocytes. This suggestion is supported by data collected by the consultant. Therefore, we have started the flow cytometry assay to detect neutrophil-ingested or monocyte-ingested reticulocytes in the spleen. The flow panel for is Ly-6C+Ly-6G+/Ly6C+Ly6G-, intracellular Ter119+ & CD71+. The analysis is pending.

### 3.2.6.2.2 RT-PCR.

At the start of H3, right and left lateral gastrocnemius muscle RNA from five control mice was generated, and the expression of two genes, *Igf-1* and *Il1b*, was assayed to test the assay protocol and form a pool of muscle RNA from control mice. No major difference in gene expression was detected between the right and left muscle RNA for both genes.

To date, 135 injured and 135 uninjured lateral gastrocnemius muscles (135 of 240 mice; 56%) have been shipped to Case Western. One sample (uninjured) was damaged during shipping. RNA extraction and cDNA synthesis have been performed on 83 injured lateral gastrocnemius muscles of 135 (61%) and 37 uninjured lateral gastrocnemius of 134 (28%) muscles. The expression levels of 13 markers have been assayed. Table 15 lists the status of these assays:

Table 15. RT-PCR Assays

Gene Symbol	Number of Injured Muscles Assayed	Number of Uninjured Muscles Assayed
<i>Slfn4</i>	80	34
<i>Stat2</i>	82	37
<i>Fst</i>	81	36
<i>Lep</i>	82	36
<i>Il10ra</i>	82	33
<i>Arg2</i>	70	22
<i>Igf1</i>	78	30
<i>Ilb</i>	78	28
<i>Tnf</i>	82	33
<i>Myf5</i>	82	33
<i>Mstn</i>	80	32
<i>Il6</i>	80	32
<i>Hif1a</i>	33	13

*Notes.* To date, the total number of injured muscles generated for RT-PCR and usable is 135, and the total number of uninjured muscles generated for RT-PCR and usable is 134. Gene symbols: *Slfn4* = schlafen 4, *Stat2* = signal transducer and activator of transcription 2, *Fst* = follistatin, *Lep* = leptin, *Il10ra* = interleukin-10 receptor antagonist, *Arg2* = arginine 2, *Igf1* = insulin like-growth factor 1, *Ilb* = interleukin-1 $\beta$ , *Tnf* = tumor necrosis factor-alpha, *Myf5* = myogenic factor 5, *Mstn* = myostatin, *Il6* = interleukin-6, *Hif1a* = hypoxia inducible factor 1 subunit alpha.

Preliminary data have been graphed for each experimental group at each time point. The mean mRNA levels of the HB mice are expressed relative to the NB mice of the corresponding group and time point (Figures 17–19). Overall, the group sizes are small. Therefore, standard deviation values are excluded, and definitive conclusions cannot be formulated at this time.

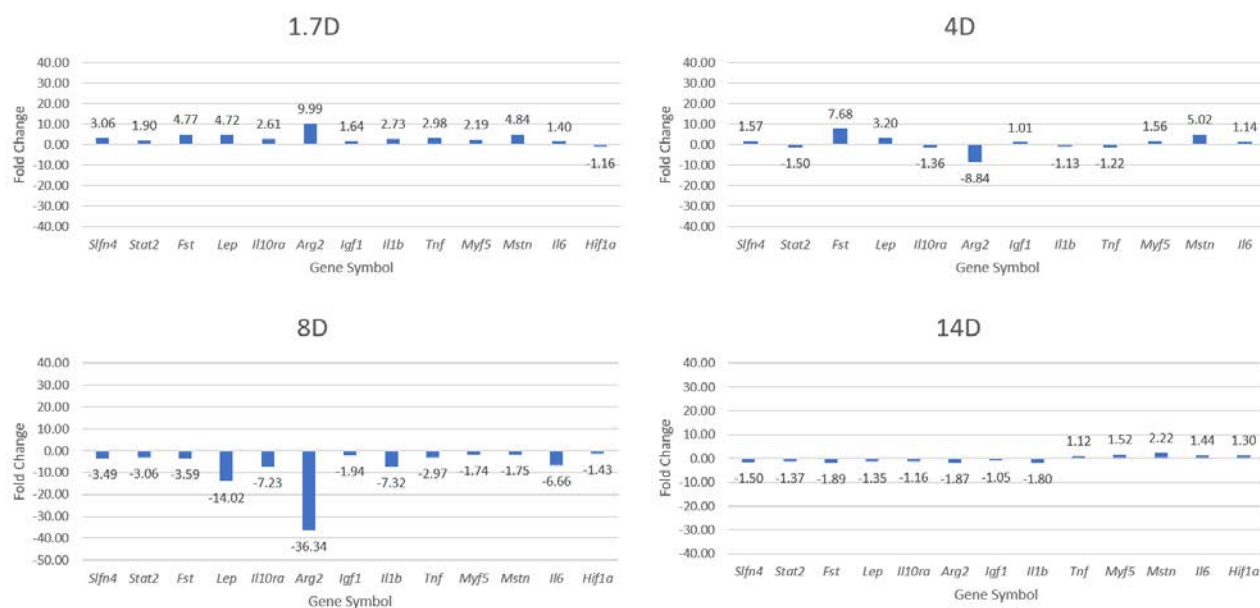


Figure 17. Hypobaric Hypoxia versus Normobaric Normoxia (Without Crush Injury or Hemorrhagic Shock) mRNA Fold Change for 13 Genes. The time point listed above each bar graph is the approximate postinjury time. The X-axis lists each of the 13 genes. Y-axis label = mRNA fold change. A fold change expression level < 1.00 indicates that the mRNA amount of that gene is expressed less in the muscle of the hypobaric hypoxia mouse compared to the normobaric normoxia mouse. A fold gene expression level > 1.00 indicates that the mRNA amount of that gene is expressed more in the muscle of the hypobaric hypoxia mouse compared to the normobaric normoxia mouse. 1.7D, 4D, 8D, and 14D = 40 hours, 4 days, 8 days, and 14 days, respectively, postinjury. Group sizes = 3–4.

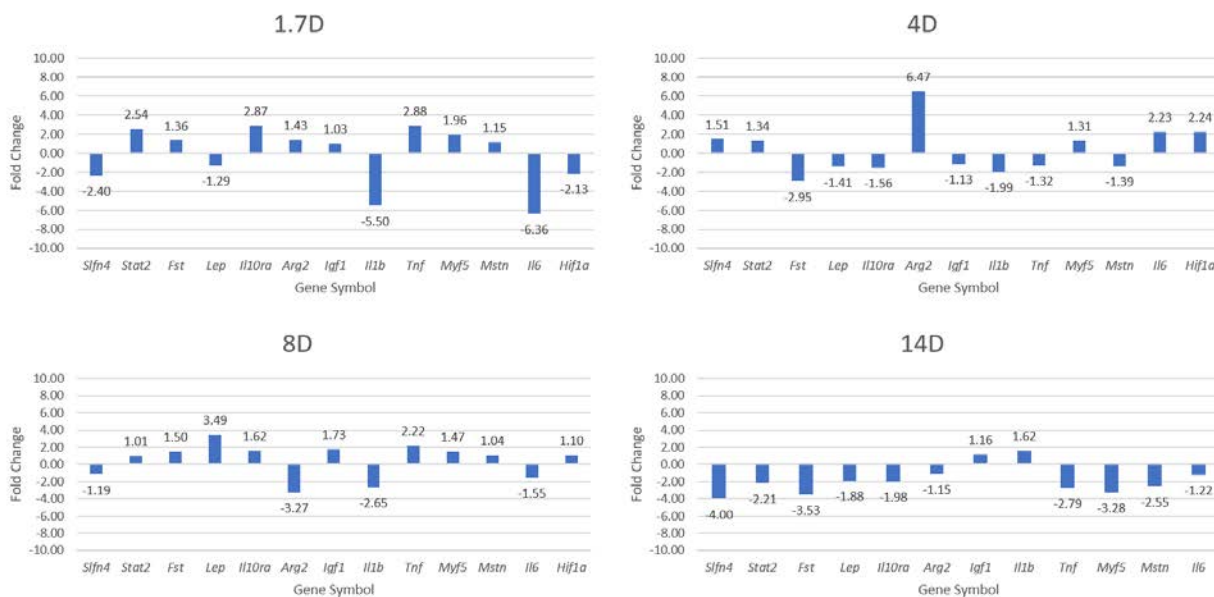


Figure 18. Hypobaric Hypoxia versus Normobaric Normoxia With Crush Injury (but Without Hemorrhagic Shock) mRNA Fold Change for 12–13 Genes. Group sizes = 2–5. The range of the y-axis label is -10.00 to +10.00, which is different from Figure 17. See Figure 17 caption for other details.

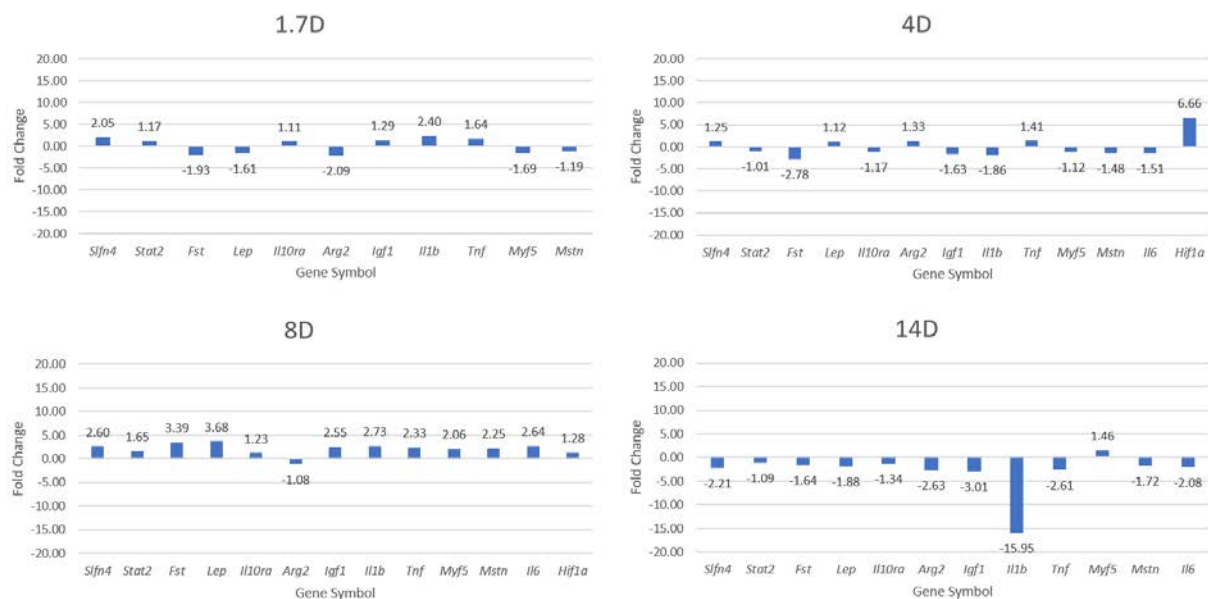


Figure 19. Hypobaric Hypoxia versus Normobaric Normoxia With Crush Injury and Hemorrhagic Shock mRNA Fold Change for 12–13 Genes. The range of the Y-axis label is –20.00 to +20.00, which is different from Figures 17 and 18. Group sizes = 2–5. See Figure 17 caption for other details.

3.2.6.2.3 Immunohistochemistry. We started immunohistochemistry late in Quarter 4. To date, 79 out of 240 (33%) of quadriceps muscles have been processed for immunohistochemistry.

3.2.6.2.4 Multiplex and ELISA. To date, sera have been collected, but no assays have been performed.

3.2.6.3 Subtask 3: Hire, orient, and train laboratory technician.

Dr. Voss completed this subtask; however, the laboratory technician resigned on 19-Jul-2019 to pursue a PhD. Therefore, a new technician will be hired as soon as possible in Year 04.

3.2.7 Major Task 2: Hypothesis 3 Testing – C57BL/6 male mice 191–380

3.2.7.1 Subtask 1: Complete animal procedures.

As indicated in Major Task 1, we have completed 259 mice. Therefore, we completed 69 mice (36%) of Major Task 2.

3.2.7.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry) mice.

Flow cytometry has begun using blood, spleen, and muscle from these mice. RT-PCR, immunohistochemistry, ELISA, and multiplex assays have not begun.

### **3.3 Training and Professional Development Opportunities**

Dr. Barbara St. Pierre Schneider, Principal Investigator

Activities completed: Attendance at two presentations given by Dr. Joan Tunnard: Cage-Side Pain Assessment in Mice and Hematology Review.

Dr. Jessica McMorris, Research Veterinarian

Activities completed: Hypobaric chamber set-up/take down, mouse monitoring during hypobaric exposure, and attendance at two presentations given by Dr. Joan Tunnard: Cage-Side Pain Assessment in Mice and Hematology Review.

Dr. Zhuowei Li, Research Technician

Activities completed: Femoral artery catheterization and induction of hemorrhagic shock with fluid resuscitation, LabChart ECG data analysis, mouse physical examination/health assessment training with Dr. Joan Tunnard, presentation of one poster at the 2018 Military Health System Research Symposium, and attendance at two presentations given by Dr. Joan Tunnard: Cage-Side Pain Assessment in Mice and Hematology Review.

Dr. Joan Tunnard, Research Veterinarian

Activities completed: UNLV IACUC and laboratory use training, mouse training (plantarflexor muscle function testing, tissue harvest, and blood collection), use of Coulter Ac•T diff2 Hematology Analyzer, hypobaric chamber set up/take down, mouse monitoring during hypobaric

exposure, creation and dissemination of two oral presentations: Cage-Side Pain Assessment in Mice and Hematology Review, mouse physical exam and health assessment discussions with Dr. Zhuowei Li and Nathan Beck, and online laboratory animal continuing education courses on Charles River Short Course.

Nathan Beck, Research Assistant

Activities completed: UNLV IACUC and laboratory use training, mouse training (plantarflexor muscle function testing, tissue harvest, tissue freezing, muscle processing for RT-PCR analysis, blood collection at euthanasia, and physical exam and health assessments), use of the Coulter Ac•T diff2 Hematology Analyzer, hypobarica chamber set up/take down, mouse monitoring during hypobarica exposure, and attendance at two presentations, Cage-Side Pain Assessment in Mice and Hematology Review given by Dr. Joan Tunnard.

Dr. Liyuan (Angi) Zhang, Postdoctoral Scholar

Activities completed: Mouse training (blood collection at euthanasia and plantarflexor muscle function testing, and muscle processing for RT-PCR analysis), use of the Coulter Ac•T diff2 Hematology Analyzer, presentation of one poster at the Military Health System Research Symposium 2018 and one poster at the 42nd Annual Conference on Shock, and attendance at two presentations given by Dr. Joan Tunnard: Cage-Side Pain Assessment and Hematology Review.

Daniela Rincon Cornejo, Project Manager

Activities completed: Attendance at two presentations given by Dr. Joan Tunnard: Cage-Side Pain Assessment and Hematology Review.

Shailey Patel, Research Assistant

Activities completed: UNLV IACUC and laboratory use training and flow cytometry assays.

Daniella Karli, Research Assistant

Activities completed: UNLV IACUC and laboratory use training, hypobarica chamber set up/take down, mouse training (tissue harvest, tissue freezing, and monitoring during hypobarica exposure), use of the Coulter Ac•T diff2 Hematology Analyzer, immunohistochemistry, H&E staining, and muscle microscopy and image analysis.

Hector Avendano-Morales, Student Worker

Activities completed: Preparation for crush injury, hemorrhagic shock, and fluid resuscitation; hypobarica chamber set up/take down; mouse monitoring during hypobarica exposure; immunohistochemistry; flow cytometry muscle processing assay; and muscle microscopy and image analysis.

Bradley Clark, Student Worker

Activities completed: UNLV IACUC and laboratory use training, hypobarica chamber set up/take down, mouse training (tissue harvest, tissue freezing, and monitoring during hypobarica exposure), use of the Coulter Ac•T diff2 Hematology Analyzer, and muscle microscopy and image analysis.

Jayla Olson, Student Worker

Activities completed: Hypobarica chamber set up/take down, mouse monitoring during hypobarica exposure), use of the Coulter Ac•T diff2 Hematology Analyzer, and muscle microscopy and image analysis.

Jazmin Lopez, Student Worker

Activities completed: Performance of multiplex (Luminex assays), use of the Coulter Ac•T diff2 Hematology Analyzer, flow cytometry instrument preparation, and muscle microscopy and image analysis.

Elena Salman, Research Assistant

Activities completed: UNLV IACUC and laboratory use training, mouse training (plantarflexor muscle function testing, tissue harvest, tissue freezing, blood collection, and monitoring during hypobarica exposure), immunohistochemistry, spleen sectioning, use of the Coulter Ac•T diff2 Hematology Analyzer, hypobarica chamber set up/take down, microscopy, and image analysis.

Olufunke Gbadamosi, Graduate Assistant

Activities completed: Performance of multiplex (Luminex assays), immunohistochemistry, H&E staining, and muscle microscopy and image analysis.

Joanna Silva, Student Worker

Activities completed: UNLV IACUC and laboratory use training; mouse monitoring during hypobarica exposure; hypobarica chamber set up/take down; use of the Coulter Ac•T diff2 Hematology Analyzer; flow cytometry blood, muscle, and spleen processing assays; and flow cytometry blood leukocyte gating.

Emma Keenan, Student Worker

Activities completed: UNLV IACUC and laboratory use training.

Karina Statkevich, Student Worker

Activities completed: Tissue harvest, tissue freezing, microscopy and image analysis.

### **3.4 Dissemination of Results**

Dissemination of results is in progress. See section 6.0, Products.

### **3.5 Year 04 Plans**

This section describes Year 04 plans for Specific Aim 1, Major Task 4: Hypotheses 1 and 2 Testing; Specific Aim 2, Major Task 2: Hypothesis 3 Testing – C57BL/6 male mice 191–380; and Specific Aim 2, Major Task 3: Hypothesis 3 Testing – C57BL/6 male mice 381–528.

#### 3.5.1 Specific Aim 1, Major Task 4: Hypotheses 1 and 2 Testing

##### 3.5.1.1 Subtask 5: Perform Hypothesis 2 analysis.

###### 3.5.1.1.1 Systemic Inflammation

Because we were unable to use the Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter) for blood leukocytes, we will consult with the biostatistician to determine whether increased group sizes are needed for the blood flow cytometry data. For the serum variables that demonstrated a trend (e.g., serum fractalkine and interleukin-16), we may request additional mice to determine whether significant differences can be detected. Furthermore, our consultant has recommended that we determine whether the immature reticulocytes observed in the spleen of hemorrhagic shock mice are undergoing proliferation or programmed cell death. We may request additional mice to perform these analyses.

#### 3.5.1.1.2 Leukocyte Populations Within the Quadriceps Muscle

We will continue to perform assays to quantify leukocyte populations within the quadriceps femoris muscle. Then, the data will be sent to Dr. Soulakova for statistical analysis.

#### 3.5.1.1.3 Plantarflexor Muscle Function

For the variables that demonstrated a trend, we may request additional mice to determine whether significant differences can be detected.

#### 3.5.1.1.4 Myofiber Regeneration in the Quadriceps Femoris Muscle

We will continue to analyze these data and then send the data to Dr. Soulakova for analysis.

#### 3.5.1.2 Subtask 6: Review Hypothesis 1 and 2 results.

We will finish reviewing Hypothesis 2 results.

#### 3.5.1.3 Subtask 7: Write and/or review report.

We plan to complete this subtask.

#### 3.5.1.4 Subtask 8: Disseminate results.

##### 3.5.1.4.1 Hypothesis 1 Manuscript

We plan to resubmit a revised manuscript to the journal, *Aerospace Medicine and Human Performance*.

##### 3.5.1.4.2 H2B Results

If we have compelling H2B results, we plan to submit a manuscript to an appropriate journal.

#### 3.5.1.5 Subtask 9: Submit and receive animal protocol amendment approval.

We may submit an animal protocol amendment to request additional mice to follow-up certain trends.

### 3.5.2 Major Task 1: Hypothesis 3 Testing – C57BL/6 male mice 1–190

#### 3.5.2.1 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry) for 190 mice.

This subtask will be completed.

### 3.5.3 Major Task 2: Hypothesis 3 Testing – C57BL/6 male mice 191–380

#### 3.5.3.1 Subtask 1: Complete animal procedures.

This subtask will be completed.

#### 3.5.3.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry).

The flow cytometry, ELISA, multiplex, and RT-PCR assays may be completed. However, the analysis may require additional time. The immunohistochemistry will be in progress.

### 3.5.4 Major Task 2: Hypothesis 3 Testing – C57BL/6 male mice 381–528

#### 3.5.4.1 Subtask 1: Complete animal procedures.

This subtask will be completed.

#### 3.5.4.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry).

The flow cytometry, ELISA, multiplex, and RT-PCR assays may be completed. However, the analysis may require additional time. The immunohistochemistry will be in progress. A no-cost extension may be requested to complete this subtask.

### 3.5.5 Strategies to Address Project Delays

As the project has been delayed for multiple reasons, the principal investigator will continue to implement strategies to ensure the entire project is completed. The strategies are as follows: (a) maintain sufficient personnel and (b) train a third staff member to perform the femoral artery catheterization, hemorrhagic shock, and fluid resuscitation procedures. In addition, we will request a no-cost extension.

## 4.0 IMPACT

### 4.1 Impact on the Development of the Principal Discipline(s) of the Project

During this year, our efforts have advanced the knowledge base regarding a combined skeletal muscle (crush) injury and hemorrhagic shock model and the differential effects of hemorrhagic shock resuscitative fluids on muscle recovery.

### 4.2 Impact on Other Disciplines

Our work regarding spleen immature reticulocytes has prompted researchers who study host defense response to burns to investigate spleen immature reticulocytes in their experimental model. To date, these researchers have presented their findings at one national conference and submitted their findings as part of one federal research grant.

Our work has also interested a researcher who studies circadian gene expression in the heart to expand into the trauma field. We have begun discussing experiments for future projects.

### **4.3 Impact on Technology Transfer**

On 25-February-2019, we submitted an application to the UNLV Office of Economic Development to determine whether our procedure for making femoral artery catheters would qualify for a utility patent. A decision is pending.

### **4.4 Impact on Society Beyond Science and Technology**

Nothing to report

## **5.0 CHANGES/PROBLEMS**

### **5.1 Changes in Approach and Reasons for Change**

#### **5.1.1 Hemorrhagic Shock and Fluid Resuscitation Model**

During the year, six major changes occurred with the model. Two changes relate to fluid resuscitation. Upon approval for an additional H2B cohort, the fluid resuscitation protocol for both H2B and H3 procedures required a modification. Specifically, no more than 1.1 ml of fluid would be administered per mouse. The rationale for this modification was to avoid fluid overload.

Based on the results of H2B, we proceeded with H3 procedures by using HEX as the resuscitative fluid. Although the use of this fluid may be limited in the clinical setting, we selected to use HEX in the current study because

- (a) hemorrhagic shock studies continue to test HEX as an alternative resuscitative fluid, and our findings can continue to provide a foundation for changes in clinical practice, and
- (b) overall, crystalloids, such as LRS, have been cited in the literature as less effective resuscitative fluids. Furthermore, with the imposed fluid restriction as explained above, there would be a concern that a return to baseline mean arterial pressure after hemorrhagic shock would be more likely with the use of LRS compared to HEX.

Another change relates to the intraperitoneal administration of HEX. We realized that HEX is labeled for intravenous administration only; therefore, we removed from the protocol the intent to administer HEX via the intraperitoneal route if the femoral artery catheter became compromised. In the current approved protocol, if the catheter is compromised and HEX is the assigned resuscitative fluid, then euthanasia will occur. If LRS is used as the resuscitative fluid (as in the case of H2B), then LRS will be administered subcutaneously.

The inclusion of subcutaneous LRS at an anesthesia maintenance rate is the third major change. To support adequate tissue perfusion postshock as much as possible, we amended the protocol and received approval so that subcutaneous LRS at an anesthesia maintenance rate could be administered immediately after fluid resuscitation if the mean arterial pressure did not return to baseline or 60 mm Hg.

Related to adequate tissue perfusion, we revised the protocol (with the required approvals) regarding temperature regulation during anesthesia. Besides changes in operating guidelines of the temperature regulation equipment, we also purchased new equipment to ensure compliance with these guidelines. We have additional protocol changes pending ACURO approval.

Additional changes included cross-training two more staff to perform the femoral artery catheterization and shock/fluid resuscitation procedure. One staff member who is full-time completed the training and has contributed greatly to our Year 02 progress for H2B and H3. A second staff member who is part-time has completed 80% of the training.

#### 5.1.2 Environmental Enrichment

We have provided enhanced environmental enrichment for mice used in this study. The standard environmental enrichment is both cotton nestlets and shredded paper. However, this level of enrichment does not seem adequate for all mice as we discovered overgrooming. Therefore, we discussed the issue with the attending veterinarian and current vendor, Charles River, and received the required approvals to provide enhanced enrichment, including an igloo and manzanita wood gnawing stick. With this new level of enrichment, the overgrooming problem has receded. We are pending ACURO approval for running wheels.

#### 5.1.3 Anticoagulant

To perform a complete blood count and flow cytometry on the same blood sample, we had to change the type of anticoagulant mixed in the blood. With the required approvals, we switched from heparin to ethylenediaminetetraacetic acid (EDTA) as recommended by the International Clinical Cytometry Society and the manufacturer of the Coulter ACT Diff 2 Analyzer (Beckman Coulter).

#### 5.1.4 Simulated Flying

Three approved changes have occurred with simulated flying. The option to use a gel-based hydration source was added to the protocol as a substitute for water bottles. Also, a procedure was added to address the situation when a water bottle leaks during simulated flying. We have extended the time interval for the flying start time from 20–28 hours to 20–30 hours. This change was needed to accommodate personnel scheduling issues, especially with the 1.7-days time point.

#### 5.1.5 Group Sizes

As indicated in the Year 02 report, our consultant recommended a 60% increase in group sizes from our proposed group sizes; however, our first step was to meet our proposed group sizes of 10 per group. (We had not met this number previously because of difficulties with the hemorrhagic shock model.) Therefore, we received approval for additional mice to achieve 10 per group. One variable that we did not achieve with 10 mice per group was blood leukocytes for flow cytometry. We removed this variable because its associated findings were neither promising nor significant, and we determined our efforts were better spent on more critical variables. However, even after removing the variable, 10 mice per group may be insufficient, and we may want to consider the consultant's recommendation of 16 per group for both H2B.

We also heeded the consultant's recommendation to follow up on the splenomegaly in the shock mice. This follow up is described in the Accomplishments section.

### 5.1.6 Plantarflexor Muscle Function Testing

Upon completion of the H2B testing, our results indicate that the testing protocol is not sufficiently intense. Therefore, with the required approvals, we increased the intensity of the testing protocol.

### 5.1.7 Cytokines

Our list of cytokines to examine in serum and muscle has changed. These changes are in response to both positive and negative findings. Specifically, we found low levels of common proinflammatory cytokines (e.g., interleukin-1) so we pursued other inflammatory cytokines. Also, we added the measurement of serum erythropoietin because of the splenomegaly. Regarding the assay of the mRNA level of muscle cytokines, we felt it would be important to detect hypoxia-inducing factor 1 to determine whether exposure to hypobaric hypoxia affected the injured muscle. The current list is described in the Accomplishments section.

### 5.1.8 Mouse Vendor

To avoid further project delays, we switched to a mouse vendor that would deliver mice during the extreme heat.

## 5.2 Changes in Personnel

Personnel changes were as follows:

5.2.1 Resignation of Dr. Andrew Murtishaw (24-August-2018)

5.2.2 Hiring and resignation of graduate assistant Olufunke Gbadamosi (20-August-2018 through 23-May-2019).

5.2.3 Hiring of Dr. Joan Tunnard (05-October-2018)

5.2.4 Hiring and resignation of staff worker Elena Salman (05-November-2018 through 30-April-2019)

5.2.5 Resignation of student worker Karina Statkevich (11-December-2018)

5.2.6 Hiring of Bradley Clark (24-January-2019)

5.2.7 Hiring of Joanna Silva (24-January-2019)

5.2.8 Hiring of Nathan Beck (01-February-2019)

5.2.9 Hiring of Daniella Karli (16-April-2019; will switch to a student worker on 26-August-2019)

5.2.10 Hiring of Kristina Mihajlovski for summer employment (28-May-2019 through 16-August-2019)

5.2.11 Hiring of Shailey Patel (13-June-2019)

5.2.12 Hiring of Emma Keenan (23-July-2019)

Note: Two personnel were on leave. One person was on approved family/medical leave for almost three months. A second person was on family/medical leave for approximately one month.

## 5.3 Actual or Anticipated Problems or Delays and Actions or Plans to Resolve the Problems

This section will focus on plans to resolve problems that are not addressed in Section 5.1: trends in H2B results and overall project delay.

While we achieved our proposed sample size for H2B with mice in Year 03, there are trends that warrant follow-up with additional mice. Our consultant also recommended a 60% higher sample size. Therefore, a discussion will be planned with the science officer regarding additional mice.

The strategies planned for Year 04 to address the overall project delay are as follows:

- a. assign a third staff person to perform femoral arterial catheterization and hemorrhagic shock and fluid resuscitations procedures,
- b. ensure that adequate staff are available to complete mouse procedures and tissue analysis,
- c. continue to cross-train personnel, and
- d. complete Hypothesis 3 animal procedures.

#### 5.4 Changes That Had a Significant Impact on Expenditures

The delay in hiring personnel has significantly affected expenditures in that year-to-date expenditures are behind. Expenditures have also been delayed because we were delayed in starting Hypothesis 2 experiments. The estimate is that the Year 03 expenditures are 6 months delayed.

#### 5.5 Significant Changes in Use or Care of Vertebrate Animals

Table 16 lists the Year 03 amendments by the UNLV IACUC-designated package number. Each amendment involved personnel changes, and other changes are described succinctly in the table. The amendment number represents a continuation from Year 02. Other related matters are stated in the table note.

Table 16. Year 03 Animal Protocol Amendments and Approval Dates

UNLV IACUC-Designated 834441 Package Number	UNLV Amendment Number	UNLV IACUC Approval Date	ACURO Amendment Number	ACURO Approval Date	Brief Amendment Description <sup>a</sup>
20	13	14-Aug-2018	13	28-Aug-2018	Changed vendor source of animals, updated mouse numbers, and clarified staff and surgical procedures and anesthesia
21	14	03-Oct-2018	14	4-Oct-2018	Minor edits
22	15	06-Nov-2018	15	7-Nov-2018	Personnel only
24	16	18-Dec-2018	16	21-Dec-2018	Added 10 control mice, transferred

					training mice, updated H3 updated to reflect H2B changes, clarified procedures, and changed cleaning and sterilization options and muscle function testing
25	17	11-Feb-2019	17	14-Feb-2019	Minor edits
27	18	29-Apr-2019	18	13-May-2019	Added drug/exogenous substance given (EDTA) and changed thermal regulation, Items of Debilitation Chart, and fluid administration
29	19	11-Jun-2019	19	pending <sup>b</sup>	Added environmental enrichment, changed food placement, and added option to monitor heart electrical activity (ECG) prior to euthanasia
30	20	03-Jul-2019	20	pending <sup>b</sup>	Added option to use gel hydration source and rodent-specific temperature feedback-controlled warming pads
32	21	24-Jul-2019	21	pending <sup>b</sup>	Added Item of Debilitation and anticipated complications

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<sup>a</sup>All amendments involved personnel changes in addition to the other changes listed in the last column.

<sup>b</sup>834441-23 was the annual UNLV IACUC continuing review/progress report. This report was submitted to ACURO on 20-December-2018.

<sup>b</sup>These and an additional document were submitted to ACURO on 25-July-2019 and 29-July-2019. ACURO approval is pending as of 16-August-2019.

<sup>c</sup>834441-26 and 834441-31 were two event reports and reported to ACURO on 26-April-2019 (first event) and 15-July-19 and 23-July-19 (second event).

<sup>d</sup>834441-28 was the result of a self-audit and revealed a protocol deviation. This self-audit was reported to ACURO on 7-May-2019 and 21-May-2019.

## 6.0 PRODUCTS

### 6.1 Publications, Conference Papers, and Presentations

We completed one podium and four poster presentations (Table 17).

Table 17. Presentations

Type	Conference	Date	Title
Poster	2018 Military Health System Research Symposium	22-August-2018	Effect of Hypobaria on Research Subjects' Well-Being and Skeletal Muscle Resident Macrophages
Poster	2018 Military Health System Research Symposium	22-August-2018	Physiological Monitoring and Intervention Setup for Experimental Hemorrhagic Shock Model for Trauma- and Immune-Related Preclinical Research
Podium	2019 Western Institute of Nursing 52nd Annual Communicating Nursing Research Conference	11-April-2019	Resuscitative Fluid Effects after Muscle Injury and Hemorrhagic Shock
Poster	Case Reserve University Inaugural Symposium for the Center for Aerospace Physiology	29-April-2019	Muscle Recovery After Lower Extremity Trauma, Shock, and Extended Mild Hypobaric Hypoxia Exposure
Poster	42nd Annual Conference on Shock	12-June-2019	Comparison of Inflammatory Responses Between Hetastarch and Lactated Ringer's Solution Treatment

### 6.2 Journal Publications

We submitted the Hypothesis 1 data as a short communication to the journal *Aerospace Medicine and Human Performance* and received a request for revision. We began revising the communication based on the reviewers' comments. (Note: We resubmitted the communication on 9-August-2019.)

### 6.3 Books or Other Non-periodical, One-time Publications

Nothing to report

#### 6.4 Other Publications, Conference Papers, and Presentations

Nothing to report

#### 6.5 Website(s) or Other Internet Site(s)

Nothing to report

#### 6.6 Technologies or Techniques

Nothing to report

#### 6.7 Inventions, Patent Applications, and/or Licenses

Nothing to report

#### 6.8 Other Products

Nothing to report

### 7.0 PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

#### 7.1 Project Members

Table 18 lists the key individuals who have worked on this project.

Table 18. Project Team Members

<b>Name</b>	<b>Dr. Barbara St. Pierre Schneider</b>
Project Role	Principal Investigator
Researcher Identifier	Not applicable
Nearest person month worked	9
Contribution to Project	Assisted with personnel training and analysis, oversaw the project by ensuring that all personnel completed assigned tasks according to deadlines and all procedures were performed according to protocols
Funding Support	Not applicable
<b>Name</b>	<b>Dr. Jessica Muniga</b>
Project Role	Research Veterinarian
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Performed mouse procedures
Funding Support	Not applicable
<b>Name</b>	<b>Dr. Zhuowei Li</b>
Project Role	Research Technician
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Performed mouse procedures and mouse data acquisition

	procedures
Funding Support	Not applicable
<b>Name</b>	<b>Dr. Liyuan (Angi) Zhang</b>
Project Role	Postdoctoral Scholar
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Performed and analyzed flow cytometry assays.
Funding Support	Not applicable
<b>Name</b>	<b>Daniela Rincon Cornejo</b>
Project Role	Project Manager
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Managed the grant budget, ordered and tracked laboratory equipment and supplies, and created/managed team members' schedules
Funding Support	Not applicable
<b>Name</b>	<b>Dr. Joan Tunnard</b>
Project Role	Research Veterinarian
Researcher Identifier	Not applicable
Nearest person month worked	6
Contribution to Project	Performed mouse procedures, assisted with animal protocol amendments, created and maintained Hypothesis 3 mouse procedure schedule, and managed mouse welfare issues
<b>Name</b>	<b>Elena Salman</b>
Project Role	Research Assistant
Researcher Identifier	Not applicable
Nearest person month worked	4
Contribution to Project	Assisted with mouse procedures and tissue analysis
<b>Name</b>	<b>Olufunke Gbadamosi</b>
Project Role	Graduate Assistant
Researcher Identifier	Not applicable
Nearest person month worked	4.5
Contribution to Project	Performed tissue analysis procedures
<b>Name</b>	<b>Hector Avendano-Morales</b>

Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	6
Contribution to Project	Performed mouse and tissue analysis procedures
<b>Name</b>	<b>Jazmin Lopez</b>
Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	6
Contribution to Project	Performed tissue analysis procedures
<b>Name</b>	<b>John Parker</b>
Project Role	Project Scientist
Researcher Identifier	Not applicable
Nearest person month worked	1
Contribution to Project	Provided consultation regarding hemorrhagic shock model and ECG analyses and performed monthly surgical instrumentation inspection
<b>Name</b>	<b>Jayla Olson</b>
Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	6
Contribution to Project	Performed tissue analysis procedures
<b>Name</b>	<b>Bradley Clark</b>
Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	4
Contribution to Project	Performed mouse procedures and tissue analysis procedures
<b>Name</b>	<b>Joanna Silva</b>
Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	4
Contribution to Project	Performed tissue analysis procedures
<b>Name</b>	<b>Daniella Karli</b>
Project Role	Student Hourly Worker

Researcher Identifier	Not applicable
Nearest person month worked	3
Contribution to Project	Performed mouse procedures and tissue analysis procedures
<b>Name</b>	<b>Karina Statkevich</b>
Project Role	Student Hourly Worker
Researcher Identifier	Not applicable
Nearest person month worked	2
Contribution to Project	Performed mouse procedures and tissue analysis procedures
<b>Name</b>	<b>Shailey Patel</b>
Project Role	Research Assistant
Researcher Identifier	Not applicable
Nearest person month worked	1
Contribution to Project	Performed tissue analysis procedures
<b>Name</b>	<b>Nathan Beck</b>
Project Role	Research Assistant
Researcher Identifier	Not applicable
Nearest person month worked	6
Contribution to Project	Performed mouse procedures and tissue analysis procedures
<b>Name</b>	<b>Emma Keenan</b>
Project Role	Student Worker
Researcher Identifier	Not applicable
Nearest person month worked	0.5
Contribution to Project	Performed mouse procedures and tissue analysis procedures
<b>Name</b>	<b>Kristina Mihajlovski</b>
Project Role	Student Worker
Researcher Identifier	Not applicable
Nearest person month worked	3
Contribution to Project	Double-checked data and schedules, and created data tables and graphs

## 7.2 Change in the Active Other Support of the PD/PI(s) or Senior/Key Personnel

None

### 7.3 Research Partners

Table 19 lists the organizations that have served as research partners.

Table 19. Research Partners

<b>Organization Name</b>	<b>University of Cincinnati</b>
Location of Organization	Cincinnati, Ohio
Partner's contribution to the project	Collaboration – review of flow cytometry results



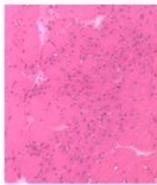
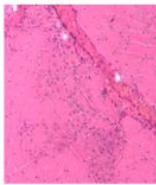
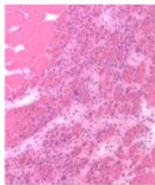
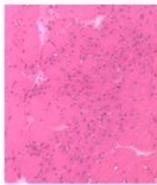
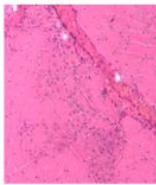
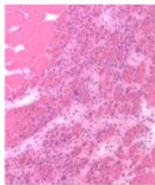
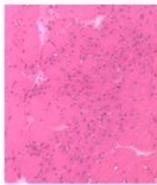
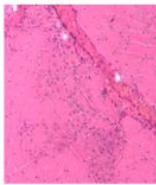
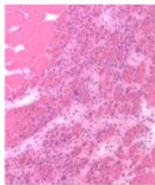
























<b>Organization Name</b>	<b>University of Central Florida</b>
Location of Organization	Orlando, Florida
Partner's contribution to the project	Collaboration – statistical analysis

<b>Organization Name</b>	<b>Case Western Reserve University</b>
Location of Organization	Cleveland, Ohio
Partner's contribution to the project	Collaboration – perform RT-PCR assays

## 8.0 SPECIAL REPORTING REQUIREMENTS

8.1 Collaborative Awards Not applicable.

### 8.2 Quad Chart

		<p>The Effect of Hypobaric on Muscle Inflammation and Regeneration after Injury and Hemorrhagic Shock JW150007, Joint Warfighter Medical Research Program</p>																															
		<p>PI: Dr. Barbara St. Pierre Schneider <b>Organization:</b> University of Nevada, Las Vegas <b>Award Amount:</b> \$ 5,558,801.00</p>																															
<p><b>Study Aim</b> The overall study aim is to examine the effect of hypobaric on muscle inflammation and regeneration after injury and hemorrhagic shock with fluid resuscitation.</p>		<p><b>Approach</b> An experimental, laboratory approach will be used to address the study aim. To examine muscle inflammation and regeneration, four major methods will be used: for proteins, immunohistochemistry flow cytometry; and for messenger RNA levels, polymerase chain reaction. Flow cytometry and serum bioassays will also be used to examine systemic inflammation. Finally, muscle function testing will be performed to examine functional recovery.</p>		<table border="1"> <thead> <tr> <th>Sham</th> <th>LRS</th> <th>HEX</th> </tr> </thead> <tbody> <tr> <td></td> <td></td> <td></td> </tr> </tbody> </table> <p>Regenerating Muscle Fibers in Quadriceps Femoris at 8 Days Postinjury. In the muscles of the Sham and LRS mice, later stage regenerating fibers are evident by large, distinct cytoplasm and central nucleus. In contrast, these fibers are difficult to observe in the muscle of the HEX mice.</p>		Sham	LRS	HEX																									
Sham	LRS	HEX																															
																																	
<p><b>Timeline and Cost</b></p> <table border="1"> <thead> <tr> <th>Activities</th> <th>17</th> <th>18</th> <th>19</th> <th>20</th> </tr> </thead> <tbody> <tr> <td>Animal Approval &amp; Hiring</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Hypothesis 1 Testing</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Hypothesis 2 Testing</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Hypothesis 3 Testing</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td><b>Estimated Budget</b></td> <td><b>\$1,271,577</b></td> <td><b>\$1,406,671</b></td> <td><b>\$1,447,661</b></td> <td><b>\$1,432,892</b></td> </tr> </tbody> </table>		Activities	17	18	19	20	Animal Approval & Hiring					Hypothesis 1 Testing					Hypothesis 2 Testing					Hypothesis 3 Testing					<b>Estimated Budget</b>	<b>\$1,271,577</b>	<b>\$1,406,671</b>	<b>\$1,447,661</b>	<b>\$1,432,892</b>	<p><b>Accomplishments:</b> Findings of the morphological assessment and flow cytometry assays of the crush-injured quadriceps femoris muscle in the hemorrhagic shock mouse model suggest that the injured quadriceps muscle takes longer to recovery after resuscitation with Hextend compared to Lactated Ringer's Solution or Sham.</p>	
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		<p><b>Goals/Milestones: CY17 and 18 Goals</b></p> <ul style="list-style-type: none"> <li><input checked="" type="checkbox"/> Animal approval, hiring personnel, and model refinement</li> <li><input checked="" type="checkbox"/> Examine effect of 16 hours of hypobaric on uninjured muscle.</li> <li><input checked="" type="checkbox"/> Set up hemorrhagic shock model.</li> <li><input checked="" type="checkbox"/> Examine inflammation activation of fluid resuscitation.</li> <li><input checked="" type="checkbox"/> <b>CY18 Goal</b> – Effect of hypobaric after injury</li> <li><input checked="" type="checkbox"/> Investigate effect of hypobaric on crush muscle injury and hemorrhagic shock in 190 subjects.</li> <li><input type="checkbox"/> <b>CY19 Goal</b> – Effect of hypobaric after injury</li> <li><input type="checkbox"/> Investigate effect of hypobaric on crush muscle injury and hemorrhagic shock in 215 subjects.</li> <li><input type="checkbox"/> <b>CY20 Goal</b> – Effect of hypobaric after injury</li> <li><input type="checkbox"/> Investigate effect of hypobaric on crush muscle injury and hemorrhagic shock in 123 subjects.</li> </ul> <p><b>Comments/Challenges/Issues/Concerns</b></p> <ul style="list-style-type: none"> <li>• Delays in animal approval, personnel hiring, setting up hemorrhagic shock/fluid resuscitation thorough reporting of our findings.</li> </ul> <p><b>Budget Expenditure to Date</b></p> <ul style="list-style-type: none"> <li><input type="checkbox"/> Projected Expenditure: \$5,558,801</li> <li><input type="checkbox"/> Actual Expenditure: \$2,886,162.24 (through 01-JUL-19)</li> </ul>																															
<p>Updated: 16-AUG-2019</p>																																	

Note: The expenditures amount provided in the July 2019 quad chart consisted of actuals + obligations. This report's quad chart reflects only actual expenditures since the project start date. We apologize for any confusion.

## **9.0 APPENDICES**

None