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14. ABSTRACT
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. To date, we have tested the hypothesis that there will be no difference in well-being and white blood cells populations in skeletal muscle between male mice exposed to hypobaria for 16 hours and male mice exposed to normobaria. This hypothesis was supported. This finding suggests that long distance flying alone does not induce obvious physiological effects or affect the presence of white blood cells normally present in skeletal muscle.

15. SUBJECT TERMS
Aeromedical evacuation
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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.

(a) 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.
(b) 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.
(c) 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 02 Project Goals

The status of the major Year 02 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. This table reflects these changes. Accomplishment details are provided in Section 3.2.

Table 1. Major Year 02 Project Goals

	Month Completed	**% Completed**
Specific Aim 1, Major Task 3: Personnel Hiring and Training		
Subtask 1: Hire personnel.	August 2017	100%

Subtask 2: Orient/train personnel as needed.	October 2017	100%
Milestones Achieved: Personnel oriented and trained.	October 2017	100%
Specific Aim 1, Major Task 4: Hypotheses 1 and 2 Testing		
Subtask 2: Perform Hypothesis 1 analysis.	September 2017	100%
Subtask 3: Set up hemorrhagic shock and fluid resuscitation model. - 40 Development Mice - 3 Refinement Model Group A Mice - 7 Refinement Model Group B Mice (These mice were planned to be used for this subtask, but because of the housing issue, we had to use these mice for crush muscle injury training.) - 36 Refinement Model Group C Mice (8 mice to optimize the crush injury protocol, and 28 mice to set up the hemorrhagic shock and fluid resuscitation model.)	March 2018	100%
Subtask 4: Perform Hypothesis 2A (20–25 C57BL/6 male mice) and 2B (75–80 C57BL/6 male mice) animal procedures.	June 2018	100%
Subtask 5: Perform Hypothesis 2 analysis.		50%
Subtask 6: Review Hypothesis 1 and 2 results.		50%
Subtask 7: Write and/or review report.		50%
Subtask 8: Disseminate results.		20%
Subtask 9: Submit and receive animal protocol amendment approval.		90%
Milestone Achieved: Hypothesis 1 and 2 testing and Animal Amendment Approval completed.		70%
Specific Aims 2 and 3: Major Task 1: Hypothesis 3 Testing – C57BL/6 male mice 1–190		
Subtask 1: Complete animal procedures.		0%
Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry) for 190 mice.	13–27	0%
Subtask 3: Hire, orient, and train laboratory technician.	13–15	0%

3.2 Accomplishment Details

3.2.1 Specific Aim 1, Major Task 3: Personnel Hiring and Training – Completed on 19-October-2017.

The newly hired postdoctoral scholar, Dr. Kelley Hammond, and two graduate assistants, Anish Puri and Elizabeth Duffy, were hired, oriented, and trained. In addition, all staff received training relevant to current procedures.

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

3.2.2.1 Subtask 2: Perform Hypothesis 1 analysis – Completed on 13-September-2017.
With the completion of the Hypothesis 1 analysis, a preliminary report was written. See Appendix A.

3.2.2.2 Subtask 3: Set up hemorrhagic shock and fluid resuscitation model – Completed on 30-March-2018.
This subtask had to be adjusted to include more mice. The reasons for this adjustment are explained in Section 5.0, Changes/Problems. This section will summarize the accomplishments.

3.2.2.2.1 Development Mice (n = 40)
These mice were completed in Year 01.

3.2.2.2.2 Refinement Model Group A Mice (n = 3)
These mice underwent Millar Mikro-Tip® catheter insertion. However, the insertion procedure was unsuccessful for all three mice, and/or the Millar Mikro-Tip® catheter broke. Three flow cytometry analyses were performed using samples from these mice. Three spleens obtained from these three mice were used to refine these steps: dissociation, cell counting, and the scatter plot.

3.2.2.2.3 Refinement Model Group B Mice (n = 7)
These mice were planned to be used for Subtask 3 and were housed individually as described in the animal protocol. However, because of the broken Millar Mikro-Tip® catheter and single housing of the mice, UNLV Institutional Animal Care and Use Committee (IACUC), Animal Care and Use Review Office (ACURO), and science officer approvals were sought (see Section 5.1.5) to use these mice for crush muscle injury practice.

The practice included subjecting the right gastrocnemius, which is one of the plantarflexor muscles, and quadriceps femoris muscle to the crush injury procedure. In addition, samples from these mice were used to assess the nature and consistency of the injury response, targeting the postinjury time points at 3 and 4 days. The injury response was primarily evaluated by examining the general morphological changes of muscle cross-sections.

In the five crush-injured gastrocnemius muscles (from five mice) used to assess the injury response, the injury response varied in magnitude from mild to moderate. However, only two of the five muscles demonstrated a moderate response, and these muscles were harvested 4 days after crush injury Figure 1).

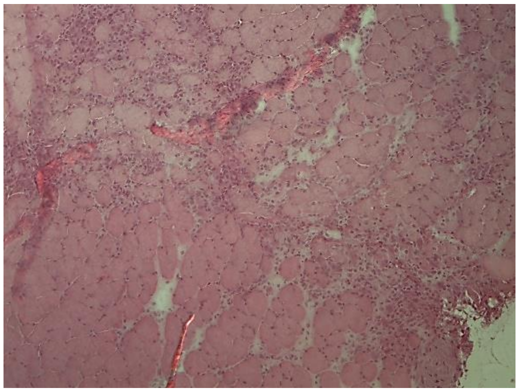


Figure 1. Moderate injury response in a plantarflexor muscle at 4 days post crush. The purple objects represent nuclei that belong to multiple cells, including macrophages and regenerating fibers. Magnification 100x.

Therefore, the injury response consisted of distinct and indistinct regenerating fibers. Specifically, the area that was commonly affected was the lateral head of the gastrocnemius muscle (LG). Also, as the thin peroneal longus (PL) muscle lies adjacent to the LG and is often dissected along with the plantarflexor muscle, an injury response was detected in one PL muscle harvested at 3 days post crush and one PL muscle harvested at 4 days post crush.

Regarding the quadriceps femoris muscle, we refined the dissection protocol, and we can successfully harvest in tandem all four muscles that compose this muscle group (Figure 2).

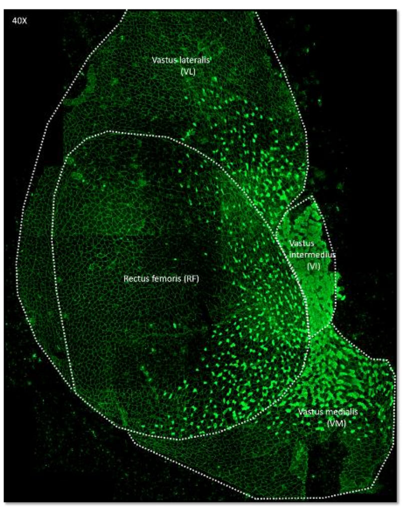


Figure 2. Four muscles of the quadriceps femoris muscle group. Vastus lateralis (top and left side), rectus femoris (center), vastus intermedius (to the right of the rectus femoris), and vastus medialis (bottom). Magnification 400x.

In terms of the injury response, two of the seven quadriceps muscles demonstrated a moderate injury response. The specific affected areas were the vastus lateralis, vastus intermedius, and/or vastus medialis. The rectus femoris did not demonstrate an injury response in any of the mice. While the nature of the injury response at 4 days is consistent with prior results, the incidence of a moderate injury response was low (~ 40%) for hypothesis testing.

We also used these mice for refine our flow cytometry assays. Spleen, whole blood, and/or uninjured and injured LG muscles were obtained from a total of four mice that underwent the crush injury procedure. Specifically, one set of uninjured and injured LG muscles from one mouse, two spleens from two mice, and pooled whole blood from three mice were used. The objectives of these assays were as follows:

(a) In whole blood, determine (i) flow cytometric parameters and (ii) Ly-6C and Ly-6G phenotypes (positive controls).
(b) In spleen preparations, determine (i) flow cytometric parameters and (ii) macrophage phenotypes (positive controls).
(c) In skeletal muscle tissue (i.e., LG-PL [LG-PL]), determine (i) flow cytometric parameters and (ii) macrophage phenotypes (experimental samples).

As depicted in Figure 3, we found the following:
(a) Approximately 8% of the whole blood cells were monocytes, and 7% were neutrophils (Figures 3A and 3B).
(b) Ninety-one percent of spleen cells expressed the CD68 antigen alone. Nearly 5% of spleen cells expressed both F4/80 and CD68 antigens.
(c) In the uninjured and crush-injured muscle preps, the percentage of CD68-positive macrophages was 8.5 and 17.6, respectively. The percentage of macrophages expressing both CD68 and CD206 antigens was 8.5% in crush-injured LG and 2.7% in uninjured LG.

These results were reviewed by and discussed with the project consultant, Dr. Caldwell.

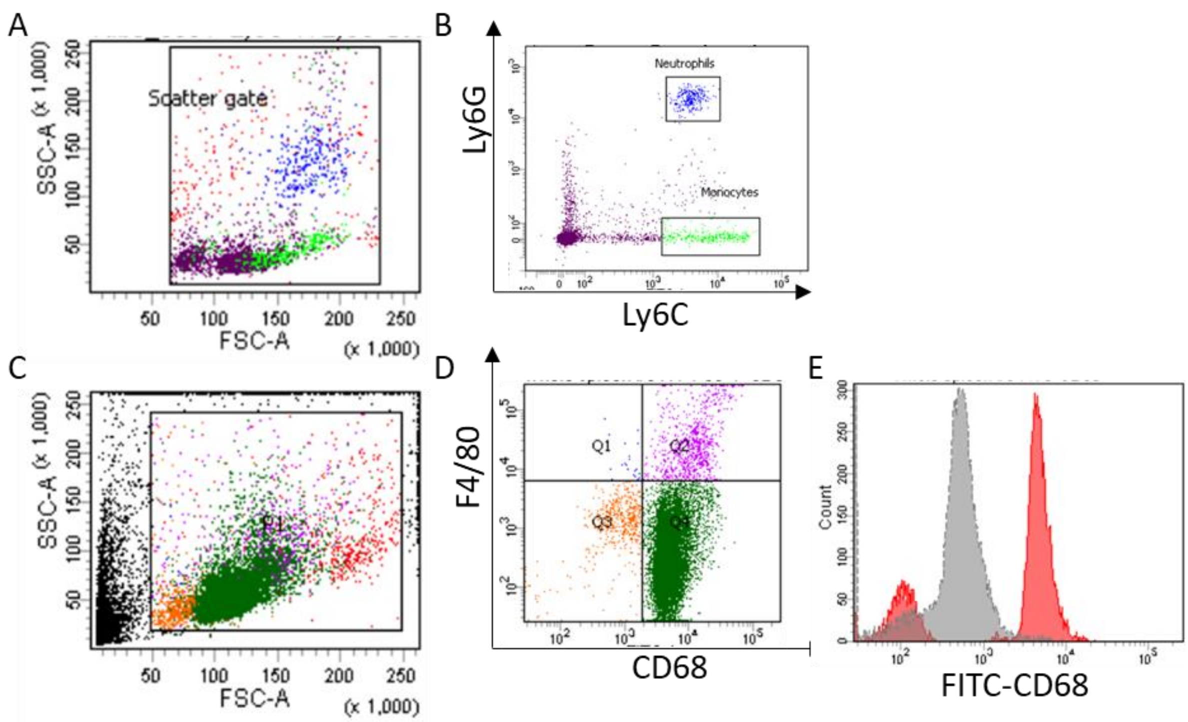


Figure 3. Flow cytometric detection of leukocyte phenotypes in whole blood and spleen. A and B: Dot plots of neutrophils and monocytes from whole blood harvested at 4 days after crush injury. Neutrophils are Ly6G+/Ly6C+ (blue color), and monocytes are Ly6G-/Ly6C+ (green color). C and D: Dot plots of spleen macrophages harvested at 3 days after crush injury. Dark green color indicates cells expressing CD68, and magenta color indicates cells expressing both CD68 and F4/80. E: Histogram of spleen CD68 (red peaks) and isotype control (grey peaks) staining. The smaller red peak is the unstained cell population, whereas the larger red peak is the stained cell population.

These findings indicate successful flow cytometric detection of leukocyte phenotypes in whole blood, spleen, and LG muscle. Specifically, we have identified neutrophils and monocytes in whole blood and macrophages in spleen and uninjured and injured muscle. However, our flow cytometric detection requires four major refinements. Our proportion of spleen CD68+ cells were higher than expected. Among spleen cells, the major leukocyte types, macrophages and lymphocytes, are approximately evenly divided, indicating that the spleen macrophage percentage should be closer to 50%. To address this issue, we should also assay spleen lymphocytes, B (CD45R/B220) and T cells (CD4 and CD8). Because LG muscle has a low number of leukocytes (in comparison to spleen), we should assay muscle for CD45, which is an antigen present on all leukocytes. A third refinement is to perform compensation tests via UltraComp™ eBeads (Grand Island, NY), which will allow the inclusion of isotype controls and reduce the overall required sample amount. Finally, lysed blood should be passed through a 40-µm strainer to remove more dead cells. With the removal of these dead cells, the cell counts in the histogram will increase. These refinements will be conducted when additional tissue becomes available.

3.2.2.2.4 Refinement Model Group C Mice (eight mice to optimize the crush injury protocol and 28 mice to set up the hemorrhagic shock and fluid resuscitation model)

3.2.2.2.4.1 Crush Muscle Injury Optimization

To address the low incidence of a moderate injury response observed in Refinement Model Group B Mice, a follow-up experiment was performed with eight mice to optimize the crush injury (see Section 5.0 regarding the related communication between the principal investigator and Science Officer, Dr. Maithili Daphtary). Optimization was required because we were using heavier male mice (closer to 30 g) for the femoral artery catheterization than the principal investigator had used in previous work. Heavier male mice could have larger muscles or more fat surrounding the muscle, which would require more crush applications or higher forces to induce a moderate injury response. We approached this optimization in a methodological way by increasing the number of applications from two to four per muscle. Eight mice were used and euthanized at four different time points post crush (Table 2). The plantarflexor muscle function testing (PMFT) was also done to evaluate whether changes of plantarflexor muscle function could be detected and whether the PMFT protocol could cause any injury to the plantarflexor muscles. The flow cytometry analysis was performed to continue to refine the flow cytometric procedures.

Table 2. Refinement Model Group C Mice (*n* = 8) for Crush Injury Optimization

EU Post crush (hours)	RG Crush	RQU Crush	PMFT	General Morphology Analysis	Flow Cytometry Analysis
2 (*n* = 2)	4x, 45 psi	4x, 45 psi	Not done	RPF – Done RQU – Done	Not done
24 (*n* = 2)	4x, 45 psi	4x, 45 psi	RPF and LPF before EU	RPF – Done RQU – Done	Blood and spleen
72 (*n* = 2)	Not done	4x, 44 or 45 psi	RPF and LPF after RQU crush	RPF – Not done RQU – Done	Not done

96 (*n* = 2)	4x, 45 psi	4x, 45 psi	RPF and LPF before EU	RPF – Not done RQU – Done	Blood, LLG, RLG, and spleen

Abbreviations: 4x = four 30 s applications of crush were delivered to the muscle; EU = euthanasia; *n* = group size; PMFT = plantarflexor muscle function testing; psi = pounds per square inch; RG = right gastrocnemius muscle; RPF = right plantarflexor muscles; and RQU = right quadriceps muscle

Regarding results, the increase in the number of applications did not increase the incidence of a moderate degree of injury as evidenced by general morphology (data not shown). The two RPF muscles that underwent plantarflexor muscle function testing (PMFT) at 72 hours before euthanasia did not demonstrate any hematomas on the muscle surface or injury in cross-section (data not shown). These data suggest that the PMFT protocol itself does not induce muscle injury. The analysis of the PMFT data is still underway.

3.2.2.2.4.2 Set Up of Hemorrhagic Shock and Fluid Resuscitation Model

The remaining 28 mice were used to set up the hemorrhagic shock model. The major result of testing these mice consisted of what we learned about the hemorrhagic shock model. That is, lowering the arterial pressure to 25 ± 5 mmHg and sustaining this pressure has a low success rate. Therefore, the principal investigator amended the animal protocol (see Section 6.0) and sought permission from Science Officer Dr. Maithili Daphtary (see Section 5.0) regarding a plan to increase the success rate. The methods and results of the approved plan are presented in 3.2.2.3.

3.2.2.3 Subtask 4: Perform Hypothesis 2A (20–25 C57BL/6 male mice) and 2B (75–80 C57BL/6 male mice) animal procedures; Subtask 5: Perform Hypothesis 2 analysis.

One hundred mice were designated to be used for animal procedures for Hypothesis 2A (H2A) and 2B (H2B). However, before the start of these procedures, seven mice sustained fighting wounds. Only three of the seven mice recovered after receiving veterinary care, and these three mice were tested as part of H2B. The H2A animal procedures and analysis will be discussed, followed by the H2B animal procedures and analysis.

3.2.2.3.1 H2A

3.2.2.3.1.1 H2A Animal Procedures

Twenty-one mice were used for H2A animal procedures, which began on 2-April-2018 and were completed on 13-April-2018. The goals included satisfactory completion and survival rates after hemorrhagic shock/fluid resuscitation. Of the 21 mice, six mice underwent crush injury and sham hemorrhagic shock; 14 mice underwent crush injury and hemorrhagic shock, followed by fluid resuscitation (Hex, *n* = 8 mice; LR, *n* = 6). One additional mouse underwent crush injury, but femoral artery catheterization was unsuccessful. For all mice, the crush injury was performed on the right hindlimb. Femoral artery catheterization for sham shock or shock mice was performed on the left hindlimb. Euthanasia of these mice was completed on 13-April-2018. Mice were scheduled to be euthanized at 1, 2, 3, or 4 days post crush.

3.2.2.3.1.2 H2A Analysis.

H2A analysis consisted of the following:

(a) Hemorrhagic Shock/Fluid Resuscitation or Sham Hemorrhagic Shock Completion and Survival Rates (see 3.2.2.3.1.2.1)

(b) Left and Right Hindlimb Recovery Rates (see 3.2.2.3.1.2.2)
(c) Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Femoris Muscles (see 3.2.2.3.1.2.3)
(d) Whole Blood Leukocyte Populations as Detected by Flow Cytometry (see 3.2.2.3.1.2.4)
(e) Plantarflexor Muscle Leukocyte Populations as Detected by Flow Cytometry (see 3.2.2.3.1.2.5)
(f) Absolute and Relative Spleen Weights (see 3.2.2.3.1.2.6)

3.2.2.3.1.2.1 H2A Hemorrhagic Shock/Fluid Resuscitation or Sham Hemorrhagic Shock Completion and Survival Rates

Table 3 shows the completion and survival rates after hemorrhagic shock/fluid resuscitation. For H2A, we concluded that we achieved a satisfactory completion and survival rates.

Table 3. H2A and H2B Hemorrhagic Shock/Fluid Resuscitation or Sham Hemorrhagic Shock Completion and Survival Rates

Event	H2A Mice Number	H2A Percent	H2B[a] Mice Number	H2B[a] Percent
Starting total mice number	21	NA	77	NA
One mouse lost[b]	NA	NA	76	NA
Successful femoral artery catheterization	20 of 21	95	75 of 76	99
Overall completion[c] (sham + shock)	17 of 20	85	72 of 76	95
Sham completion[c]	6 of 6	100	23 of 24	96
Shock completion[c]	11 of 14	79	49 of 52	94
Survival[d]	15 of 17	88	70 of 72	97
Survival until scheduled euthanasia following shock or sham	14 of 17	82	69 of 72	96
Comorbidities discovered during euthanasia	0	0	67 of 72	93

Note: NA = not applicable. [a]The two mice transferred from H2A are included in these data. [b]One mouse had to be euthanized because after crush injury—but before femoral artery catheterization—a large back wound was discovered. [c]Completion = The mouse underwent crush injury followed by sham shock or shock/fluid resuscitation as described in the animal protocol and recovered from anesthesia without experiencing major respiratory problems. [d]Survival = The mouse survived for more than 19 hours after crush injury followed by sham shock or shock/fluid resuscitation.

3.2.2.3.1.2.2 H2A Left and Right Hindlimb Recovery Rates

This project represented the first time we have (a) combined closed crush muscle injury with femoral artery catheterization, hemorrhagic shock, and fluid resuscitation and (b) used male mice ranging in age from 21–23 weeks with a body weight ranging from 27–33 g. Because of their age and body weight and the goal to yield a moderate degree of crush injury, the majority of the mice received a crush injury stimulus consisting of 50 psi and four applications each to the gastrocnemius and quadriceps muscles. While a satisfactory left hindlimb recovery rate (Table 4) was yielded, we determined that 50 psi and four applications to each muscle may have contributed to right hindlimb issues (e.g., tibia fracture, compartment syndrome, or nerve damage).

Table 4. H2A and H2B Left and Right Hindlimb Recovery Rates

Recovery Rate	H2A Mice Number	H2A Percent	H2B Mice Number	H2B Percent
Left hindlimb	16 of 17	94	70 of 72	97
Right hindlimb	11 of 17	65	71 of 72	99

Note: Left hindlimb recovery rate = The number of mice that completed crush injury and sham shock or shock and demonstrated no overt left hindlimb mobility or nerve issues. Right hindlimb recovery rate = The number of mice that completed crush injury and sham shock or shock and demonstrated no overt right hindlimb mobility, bone, or nerve issues.

3.2.2.3.1.2.3 H2A Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Muscles

We evaluated the occurrence of hematomas in crush-injured muscles of 17 mice. Of these mice, 100% and 94% exhibited a hematoma in the crush-injured gastrocnemius and quadriceps muscles, respectively. These findings indicate that an adequate crush injury occurred to these muscles.

3.2.2.3.1.2.4 H2A Whole Blood Leukocyte Populations as Detected by Flow Cytometry

Whole blood (*n* = 7 mice) underwent flow cytometry to detect specific leukocytes expressing the proteins, CD11b, Ly-6G, and Ly-6C. Combination of the differential expression of these proteins informs us whether neutrophils and/or monocytes are present and/or exhibit a change in proportion. On 8-May-2018, we met with the consultant, Dr. Caldwell, via teleconference to review the results via teleconference. The results of this call led to more technical refinements.

3.2.2.3.1.2.5 H2A Plantarflexor Muscle Leukocyte Populations as Detected by Flow Cytometry

Through our preliminary flow cytometry assays, we learned that our cell count yield would be adequate for flow cytometry by assaying the entire plantarflexor muscle instead of only the LG muscle, which is one of the four muscles of the plantarflexor muscle group. Uninjured and injured plantarflexor muscles (*n* = 6 mice) underwent flow cytometry to detect macrophages, eosinophils, and other granulocytes. On 8-May-2018, we met with the consultant, Dr. Caldwell, via teleconference to review the results. The results of this call led to more technical refinements.

3.2.2.3.1.2.6 H2A Absolute and Relative Spleen Weights

As immune system changes can be evident in the spleen, the spleen was harvested immediately after euthanasia and weighed to determine the absolute spleen weight. We also calculated the relative spleen weight, which is the absolute spleen weight (mg) divided by the mouse body weight (g). The relative spleen weight is listed by group in Table 5. The data suggest that the relative spleen weight of shock mice is increased by almost two fold in comparison with sham shock mice. The mouse with the highest relative spleen was 4 days post crush. This time point is of interest because 4 days after crush injury represents the start of muscle regeneration after an acute injury. Dr. Caldwell indicated that the increase in spleen weight reflects that the addition of hemorrhagic shock induces a more severe injury than crush injury alone. Also, he suggested 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.

Table 5. Relative Spleen Weight for H2A Mice

Group	Range	Median
Sham Shock (*n* = 5 mice)	2.42–3.11 mg/g	2.88 mg/g
Shock (*n* = 8 mice)	1.52–7.71 mg/g	5.00 mg/g

Note: These mice were euthanized approximately 1–4 days after sham or shock.

3.2.2.3.2 H2B

3.2.2.3.2.1 H2B Animal Procedures
The H2B animal procedures began on 16-April-2018 and were completed on 22-June-2018. The H2B hypothesis is mice that undergo crush muscle injury and hemorrhagic shock, followed by LR fluid resuscitation, will demonstrate a slower muscle recovery and greater systemic inflammation than that of mice that undergo crush muscle injury and hemorrhagic shock, followed by Hex resuscitation.

Seventy-seven mice were used (which includes two mice from H2A). The specific details regarding completion, survival, and other issues are listed in Table 3. In the end, 67 mice survived and underwent tissue analysis. At the time of crush injury, the age and body weight range of the mice was 10–22 weeks and 24–29 g, respectively. The crush stimulus consisted of 45 psi, except for four mice (40 psi for one mouse; 50 psi for three mice). The gastrocnemius muscle of 66 mice received 2–4 applications. The gastrocnemius muscle of one mouse received only one application. The quadriceps muscle of 67 mice received 3–4 applications. Table 6 lists the group sizes.

Table 6. H2B Groups

EU Time Point Days	Sham	Hex	LR	Total Mouse Number
1.5	7	8	8	23
4	8	8	8	24
8	8	6	6	20
Total Mouse Number	23	22	22	67

Abbreviations: EU = euthanasia; Hex = Hextend; LR = lactated Ringer’s
Note: All mice underwent crush injury of the right gastrocnemius and quadriceps muscles as well as left femoral artery catheterization. The mice in the LR 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.

3.2.2.3.2.2 H2B Dependent Variables and Analysis
Based on our experience with H2A, the following three dependent variables were examined:

(a) H2B Hemorrhagic Shock/Fluid Resuscitation or Sham Hemorrhagic Shock Completion and Survival Rates (see 3.2.2.3.2.2.1)
(b) Left and Right Hindlimb Recovery Rates (see 3.2.2.3.2.2.2)
(c) Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Femoris Muscles (see 3.2.2.3.2.2.3)

In addition, the examination of these five proposed dependent variables is in progress:

(a) Systemic Inflammation (see 3.2.2.3.2.2.4)
(b) Leukocyte Populations Within the Lateral Gastrocnemius or Plantarflexor Muscles (3.2.2.3.2.2.5)
(c) Leukocyte Populations Within the Quadriceps Femoris Muscle (see 3.2.2.3.2.2.6)
(d) Plantarflexor Muscle Function (see 3.2.2.3.2.2.7)
(e) Myofiber Regeneration in the Quadriceps Femoris Muscle (see 3.2.2.3.2.2.8)

These variables are discussed separately in the following subsections.

3.2.2.3.2.2.1 H2B Hemorrhagic Shock/Fluid Resuscitation or Sham Hemorrhagic Shock Completion and Survival Rates

Table 3 shows that we achieved an adequate completion rate of hemorrhagic shock/fluid resuscitation and an adequate survival rate after fluid resuscitation. Interestingly, of the total 10 mice that were not used (out of 77), five had received Hex and developed issues post catheterization.

3.2.2.3.2.2.2 H2B Left and Right Hindlimb Recovery Rates

As indicated in Table 4, satisfactory right and left hindlimb recovery rates were achieved.

3.2.2.3.2.2.3 H2B Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Femoris Muscles

We evaluated the occurrence of hematomas in crush-injured gastrocnemius (*n* = 67) and quadriceps femoris (*n* = 66) muscles (preliminary results, Table 7). Hematomas were present at 1.5 and 4 days post crush, and occurrence was decreased at 8 days post crush in all groups. One qualitative observation is that the hematomas in the injured gastrocnemius muscles of Hex-resuscitated mice tended to be darker in color than those in the LR-resuscitated or sham mice (Figure 4).

Table 7. H2B Hematoma Occurrence in Crush-injured Gastrocnemius and Quadriceps Femoris Muscles

	Crush-injured Gastrocnemius			Crush-Injured Quadriceps Femoris		
EU Time Point Days	Sham	Hex	LR	Sham	Hex	LR
1.5	100%	100%	100%	86%	100%	100%
4	88%	100%	100%	88%	100%	88%
8	63%	50%	33%	63%	60%	50%

Abbreviations: EU = euthanasia; Hex = Hextend; and LR = lactated Ringer's

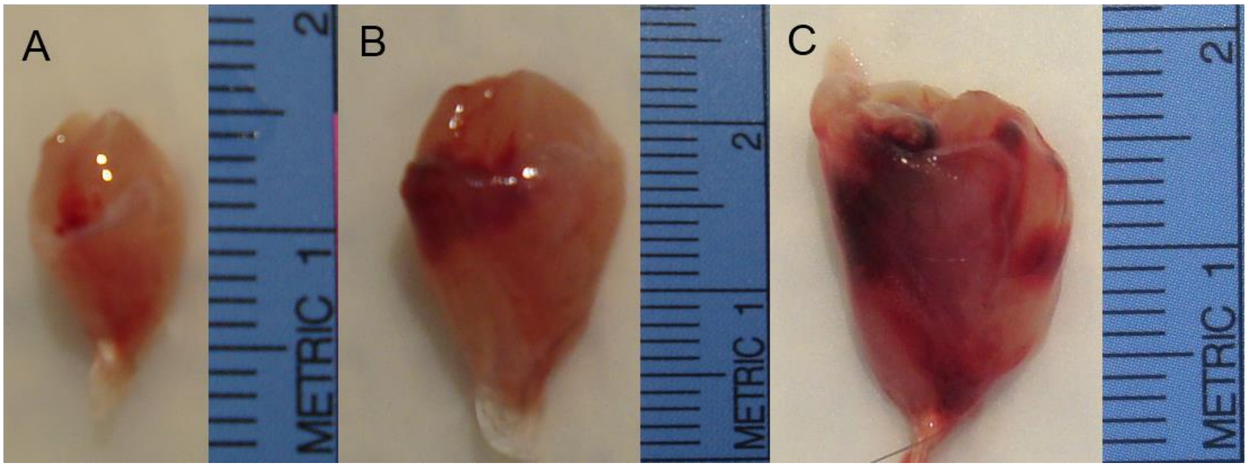


Figure 4. H2B – Hematoma presence in gastrocnemius muscle at 1.5 days post crush. Two of the hematomas look darker in the crush-injured gastrocnemius muscle of the Hex-resuscitated mouse (C) than those in the sham shock (A) and LR-resuscitated (B) mice. Hex = Hextend; and LR = lactated Ringer's

3.2.2.3.2.2.4 Systemic Inflammation

In the proposal, this dependent variable is divided into two indicators: whole blood leukocyte populations and serum concentrations of pro-inflammatory 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.

3.2.2.3.2.2.4.1 Whole Blood Leukocyte Populations

We detected whole blood leukocyte populations using flow cytometry. Sixty-four mice were used. Although we had proposed to detect T cells, B cells, and eosinophils, we had to omit this detection because of time constraints. However, we detected the total number of blood leukocytes, blood neutrophils, and blood monocytes. Preliminary results are indicated in Figure 5. Preliminary statistical analysis revealed (a) a significant difference in the total blood leukocytes and blood monocyte counts and (b) higher counts of these cells in the LR-resuscitated group than in the sham shock group. No significant difference in blood neutrophil count was detected (data not shown).

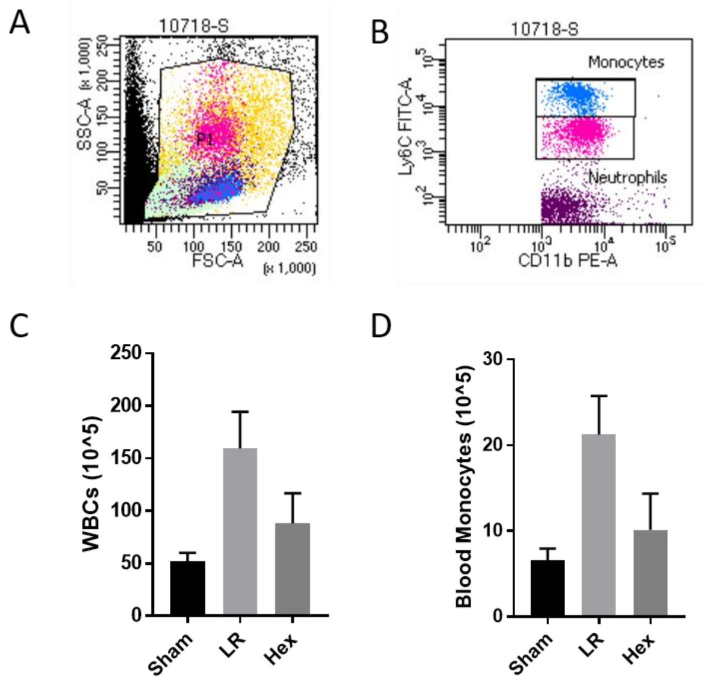


Figure 5. Flow cytometry of whole blood leukocyte populations. A: Dot plot of leukocytes obtained from whole blood of a mouse euthanized after 4 days of crush injury and no shock. Among the leukocytes are neutrophils (magenta population) and monocytes (blue population). B: From dot plot in A, neutrophils are CD11b+/Ly6C- (magenta color), and monocytes are CD11b+/Ly6C+ (blue color). C: Bar graph of the blood leukocyte (abbreviated WBCs) count obtained from mice after 4 days of crush injury with and without shock/fluid resuscitation. The total WBC count of LR-resuscitated mice is significantly higher than that of sham mice (pairwise comparison, p = 0.03). D. Bar graph of the blood monocyte count obtained from mice after 4 days of crush injury with and without shock/fluid resuscitation. The blood monocyte count of LR-resuscitated mice is significantly higher than that of sham mice (pairwise comparison, p = 0.03). Values are means ± standard error. Sham group (*n* = 6), LR group (*n* = 7), and Hex (*n* = 6) group. Abbreviations: Hex = Hextend; LR = lactated Ringer's

Although Dr. Caldwell's consultation occurred on 30-July-2018 and 31-July-2018, which is past the period of this report, the results of this consultation are salient to discuss in this report. After careful and thoughtful review of the blood leukocyte flow cytometry data, a preliminary conclusion was drawn regarding the two types of fluid resuscitation: Hex and LR. The fluids, Hex and LR, appear to affect the blood leukocyte response differentially. This trend is hinted in Figure 5. Based on this trend and his flow cytometry expertise, Dr. Caldwell concludes that a total of 16 mice per group per time point is needed to test Hypothesis 2. To achieve this group size, an additional eight to ten mice per group per time point will be needed, totaling 82 mice. However, with the UNLV School of Nursing's recent acquisition of the Beckman Coulter AcT Diff 2 hematology analyzer, Dr. Caldwell states that this instrument could be used to generate these findings, which would limit reagent costs and reduce processing time. Furthermore, these mice could be used to booster the findings of the plantarflexor muscle leukocyte populations as detected by flow cytometry (see 3.2.2.3.2.2.5 below). One additional recommendation related to the blood leukocytes is to perform an ex vivo experiment to determine whether blood monocytes are being primed (to be prepared for activation) by LR.

3.2.2.3.2.2.4.2 Serum Concentrations of Pro-inflammatory Cytokines, Anti-inflammatory Cytokines, and Chemokines

Our progress to date is that the serum was collected. The analysis of this serum was affected in part by the resignation of Dr. Hammond. The serum concentrations will be determined during Year 03, Quarter 1.

3.2.2.3.2.2.4.3 Median Absolute and Relative Spleen Weights

Based on our H2A observation that shock mice may experience an enlarged spleen, we weighed the spleens for all 67 mice and calculated the median absolute and relative spleen weights (preliminary results, Table 8). As Dr. Caldwell is an expert in spleen immunology, these data were reviewed during his consultation visit on 30-July-2018 and 31-July-2018. According to Dr. Caldwell, the sham spleen weights at all time points are lower than normal, which reflects an immunologic response to the crush muscle injury alone. Regarding the elevated spleen weights in the resuscitated groups at 4 and 8 days, these findings indicate that the addition of hemorrhagic shock induces a more severe injury than crush injury alone. Also, Dr. Caldwell 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 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. Dr. Caldwell also recommended that the function of spleen cells be tested to determine whether the resuscitation fluids affect the spleen cells.

Table 8. Spleen Weight for H2B Mice

	Median Absolute Spleen Weight (mg)			Median Relative Spleen Weight (mg/g)		
EU Time Point Days	Sham	Hex	LR	Sham	Hex	LR
1.5	51.00	56.95	60.50	2.13	2.52	2.63
4	66.95	138.05	183.60	2.75	5.88	7.38
8	67.10	97.90	102.30	2.59	3.80	3.90

Abbreviations: EU = euthanasia; Hex = Hextend; LR = lactated Ringer’s

3.2.2.3.2.2.5 Leukocyte Populations within the Plantarflexor Muscles

Another dependent variable is the leukocyte populations within the plantarflexor muscles. In the proposal, we planned to perform flow cytometry to assay the leukocytes within the lateral gastrocnemius muscle, which is one of the four muscles composing the plantarflexor muscle group. However, because the cell yield was too low for adequate flow cytometry, we had to include all plantarflexor muscles.

Flow cytometry of plantarflexor muscle leukocyte populations was completed using all 67 mice. During H2A, we learned that converting a whole muscle into a single cell suspension is a three-hour procedure. Then, the actual flow cytometry of this single cell suspension takes several more hours. Because of the intensive time requirement of flow cytometry and our flow cytometry capability of only two lasers, we focused our H2B efforts on detecting M1 macrophages, M2 macrophages, and eosinophils. We did not detect T and B cells.

These data were carefully and intensely reviewed during Dr. Caldwell's consultation on 30-July-2018 and 31-July-2018. Similar to the blood flow data (as described in 3.2.2.3.2.2.4.1), Dr. Caldwell observed possible trends in the data, including that fluid resuscitation type affects the muscle leukocyte populations. Two specific trends to consider are (a) a higher number of eosinophils after resuscitation with LR than with Hex and (b) a higher number of M2 macrophages, which are associated with an anti-inflammatory response, with Hex than with LR. Dr. Caldwell found these trends of significant interest and advised us to pursue these trends in three major ways:

(a) reanalyze the existing data by focusing on M2 macrophages;
(b) continue the experiment and achieve a total of 16 mice per group per time point to ensure an adequate number of mice per group for statistical analysis; and
(c) with the serum collected from these mice, perform assays targeting chemokines that specifically attract eosinophils and M2 macrophages to substantiate the increased infiltration of eosinophils and M2 macrophages.

3.2.2.3.2.2.6 Leukocyte Populations Within the Quadriceps Femoris Muscle

Analysis of the leukocyte populations within the quadriceps femoris muscles has started but has proceeded more slowly than anticipated because Dr. Hammond resigned in June. To address this change in personnel, Dr. Schneider started sectioning the remaining muscles. Almost 50% of the muscles have been cut into 10-micron thick cross-sections.

We have examined the general morphology of sections obtained from mice euthanized at 1.5 days post injury. Because the injured fibers (as evidenced by pale eosin staining) looked smaller in size in the muscle of the sham shock mice compared to that of the shock mice, we developed a protocol to measure the cross-sectional area of these fibers. As part of this protocol, the number of internal nuclei and gaps within the fibers are also quantified. One interesting trend is that the total number of pale fibers is greatest in the muscle of the Hex-resuscitated group in comparison with that of the LR-resuscitated and sham shock groups. However, more muscles need to be analyzed.

As proposed, we are immunolabeling the sections for CD68-positive, CD206-positive, and F4/80-positive cells. To compare with the flow data, we added immunolabeling for CD11b-positive cells. At this point, we have no data or trends to report.

3.2.2.3.2.2.7 Plantarflexor Muscle Function

As described in the proposal, muscle function testing was conducted on plantarflexor muscles immediately before euthanasia. We have usable data from 63 of 67 mice and have started to process the data. These data include isometric torque before and after 50 eccentric contractions and eccentric torque during the 50 eccentric contractions.

3.2.2.3.2.2.8 Myofiber Regeneration in the Quadriceps Femoris Muscle

Analysis of myofiber regeneration within the quadriceps femoris muscles harvested 4 and 8 days post injury (n = 44) has begun. However, the analysis has proceeded more slowly than anticipated because of Dr. Hammond's resignation in June. To address this change in personnel, Dr. Schneider started sectioning the remaining muscles. Approximately 50% of the muscles have been cut into 10-micron thick cross-sections.

We have assessed the presence of regenerative fibers qualitatively using hematoxylin-eosin-stained cross-sections. Since writing the proposal, we have discovered that at 4 days post crush injury, two types of regenerating fibers are present: distinct and indistinct. Our assessment has included identifying these areas.

3.2.2.4 Subtask 6: Review Hypothesis 1 and 2 results.
The review of Hypothesis 1 results was completed on 22-September-2017.
We estimate completing this task for Hypothesis 2B by the end of Year 03, Quarter 1.

3.2.2.5 Subtask 7: Write and/or review report.
We completed and submitted a copy of the Hypothesis 1 to Science Officer Dr. Shelley Jorgensen on 26-September-2017. A copy is also included in Appendix A. Based on the 67 H2B mice, we estimate completing Subtask 7 by the end of Year 03, Quarter 2.

3.2.2.6 Subtask 8: Disseminate results.
We still plan to submit the Hypothesis 1 results to the journal, *Nursing Research*. We submitted two abstracts to the 2018 Military Health System Research Symposium, and both abstracts were accepted for poster presentations. The abstract titles are as follows:

> Effect of Hypobaria on Research Subjects' Well-Being and Skeletal Muscle Resident Macrophages
>
> Physiological Monitoring and Intervention Setup for Experimental Hemorrhagic Shock Model for Trauma- and Immune-Related Preclinical Research

If we have compelling Hypothesis 2 results, we plan to present these results at a trauma-related or physiology-related conference.

3.2.2.7 Subtask 9: Submit and receive animal protocol amendment approval.
We submitted and received animal protocol amendment approval for seven amendments. See Section 6.0 for details.

3.3 Training and Professional Development Opportunities
Dr. Jessica Muniga, Research Veterinarian
Activities completed: Additional two-day mouse femoral artery catheterization and hemorrhagic shock training at UNLV

Dr. Zhuowei Li, Research Technician
Activities completed: Two-day mouse femoral artery catheterization and hemorrhagic shock training at the UCLA Mouse Physiology Laboratory.

Dr. Kelley Hammond: Postdoctoral Scholar
Activities completed: IACUC training, mouse training (including harvesting tissue and blood collection), immunohistochemistry, cryosectioning, data organization and rigor, and imaging analysis software

Daniela Rincon Cornejo, Project Coordinator
Activities completed: UNLV purchasing and accounting software, UNLV Human Resources procedures, and group facilitation training

3.4 Dissemination of Results
Dissemination of results is in progress. See 3.2.2.6, Subtask 8.

3.5 Year 03 Plans
This section describes Year 03 plans for Specific Aim 1, Major Task 4: Hypotheses 1 and 2 Testing and Specific Aim 2, Major Task 1: Hypothesis 3 Testing – C57BL/6 male mice 1–190.

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
We plan to assay the H2B mouse serum for systemic inflammation, detecting pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines. In performing these assays, Dr. Caldwell advised us to start with the chemokines and adjust our chemokine panel to include granulocyte-colony stimulating factor, monocyte-derived chemokine, eotaxin, chemokine ligand 17 (TARC), granulocyte-macrophage colony stimulating factor, and high mobility group box 1 protein. We anticipate that the time required to complete these assays is 1–2 months. Then, the next step is statistical analysis performed by Dr. Soulakova.

3.5.1.1.2 Leukocyte Populations Within the Plantarflexor Muscles
Based on Dr. Caldwell’s recommendation, we plan to reanalyze the current H2B muscle flow data to focus on the M2 macrophages. We anticipate that the time required will be 2–3 days. After this analysis, we will consult with Dr. Caldwell on 11-September-2018 regarding the interpretation of the results and the flow cytometry panel for Hypothesis 3. The next step is statistical analysis of the data, which will be performed by Dr. Soulakova.

3.5.1.1.3 Leukocyte Populations Within the Quadriceps Muscle
We will continue to perform assays to quantify leukocyte populations within the quadriceps muscle by the end of Year 03, Quarter 1. Then, the data will be sent to Dr. Soulakova for statistical analysis.

3.5.1.1.4 Plantarflexor Muscle Function
The next step is for the principal investigator to review the data and distribute the data for statistical analysis, which will be performed by Dr. Soulakova.

3.5.1.1.5 Myofiber Regeneration in the Quadriceps Femoris Muscle
We will continue to perform assays to determine whether myofiber regeneration in the quadriceps muscle differs among sham shock, Hex-resuscitated, and LR-resuscitated mice. We anticipate completing this work by the end of Year 03, Quarter 1. The data will be sent to Dr. Soulakova for statistical analysis.

3.5.1.1.6 Other Hypothesis 2 Plans

3.5.1.1.6.1 Spleen Myelopoiesis
Based on Dr. Caldwell's advice, we plan to verify the presence of myelopoiesis in the spleen of mice euthanized 4 days post injury. We saved four spleens to perform this analysis. We anticipate that the time required to complete this analysis is one week.

3.5.1.1.6.2 Increased H2B Group Sizes
Dr. Caldwell's advice to increase group sizes must be seriously considered. The principal investigator plans to discuss this advice with the science officer.

3.5.1.2 Subtask 6: Review Hypothesis 1 and 2 results.
We plan to complete this subtask in Year 03.

3.5.1.3 Subtask 7: Write and/or review report.
We plan to complete this subtask in Year 03.

3.5.1.4 Subtask 8: Disseminate results.

3.5.1.4.1 Hypothesis 1 Manuscript. Although we have been delayed in submitting the Hypothesis 1 manuscript, we still plan to submit this manuscript for publication. In the quarterly reports, we have mentioned the need to include more data. When time permits, we have been working on this task.

3.5.1.4.2 H2B Results
If we have compelling H2B results, we plan to submit abstracts to a nursing research conference and the Shock Society annual conference.

3.5.1.5 Subtask 9: Submit and receive animal protocol amendment approval.
As a result of performing Hypothesis 2, we need to make minor changes in the animal protocol for Hypothesis 3. The plan is to submit this protocol to the UNLV IACUC late August or early September 2018. Then, the approved protocol will be submitted to ACURO.

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

3.5.2.1 Subtask 1: Complete animal procedures.
We anticipate starting Hypothesis 3 in October 2018. With a second staff member performing the femoral artery catheterization, hemorrhagic shock, and fluid resuscitation (see 3.5.3.2 below), the possibility exists that this subtask may be completed by the end of Year 03.

3.5.2.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry) for 190 mice.
This subtask may be completed by the end of Year 03.

3.5.2.3 Subtask 3: Hire, orient, and train laboratory technician.
Dr. Voss plans to complete this task in Year 03, Quarter 1.

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 may be completed by the end of Year 03.

3.5.3.2 Subtask 2: Complete flow cytometry, immunohistochemistry, ELISA, multiplex, and RT-PCR assays (includes data entry) for 380 mice.
This subtask may be completed by the end of Year 03.

3.5.4 Other Plans.

3.5.4.1 Personnel Hiring
During Year 03, Quarter 1, more personnel will be hired. With the departure of Dr. Hammond, another postdoctoral scholar or a research technician will be hired. However, we may hire both personnel (see 3.5.5). We were unable to fill one of the graduate assistantships, so these funds may be used to support a research technician or additional student workers.

3.5.4.2 Strategies to Address Project Delays
As the project has been delayed for multiple reasons, the principal investigator would like to implement strategies to ensure that the entire project is completed. The strategies are as follows: (a) hire additional personnel if the budget permits and (b) train a current second staff member to perform the femoral artery catheterization, hemorrhagic shock, and fluid resuscitation procedures. Both the UNLV IACUC and ACURO have approved the use of 20 mice for training this staff member.

4.0 IMPACT

4.1 Impact on the Development of the Principal Discipline(s) of the Project
During this year, our efforts have resulted in the development of a preclinical mouse model that can be used to study skeletal muscle inflammation and regeneration after a closed crush injury followed by hemorrhagic shock/fluid resuscitation. This model can be used to test interventions to improve the recovery of civilians and military personnel who sustain both muscle trauma and hemorrhage.

4.2 Impact on Other Disciplines
Nothing to report

4.3 Impact on Technology Transfer
Nothing to report

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 Year 02, major changes in our approach centered on the hemorrhagic shock and fluid resuscitation model. Originally, we had proposed to insert an arterial catheter system composed of a Millar Mikro-Tip® arterial pressure sensing catheter attached to adaptors to remove blood and instill fluid. However, we learned that this system would not be feasible for the mouse femoral artery, so we changed the approach to insert the Millar Mikro-Tip® catheter into one femoral artery and then insert a second catheter in the other femoral artery to remove blood and instill fluids. In testing this approach, we encountered that the Millar Mikro-Tip® catheter was prone to breakage and too flexible to achieve a high catheter insertion rate (despite additional training, additional surgical instruments, and the use of heavier mice). Therefore, we changed the catheter system again to a polyethylene tubing with an external transducer and ports so arterial pressure could be monitored, blood removed, and fluids instilled. As discussed and approved by the Science Officer, Dr. Maithili Daphtary (during the period of February 2018 and March 2018), we tested this system with 28 nonsurvival mice (referred to as Refinement Model Group C), followed by 21 survival mice (referred to as H2A mice). As shown for our Hypothesis 2 results (H2A and H2B) results, our mouse completion and survival rates indicate that this system works.

We also made other changes to the model to improve our monitoring of the mouse and achieve adequate mouse completion and survival rates. Specifically, we added a cautery unit to help control excessive bleeding during the catheterization, an electrocardiogram lead system to monitor heart rate, a rectal temperature probe to monitor body temperature, and heat lamps to ensure an adequate body temperature during anesthesia. We changed the target arterial blood pressure from 25 ± 5 mmHg to 25–45 mm Hg.

5.1.2 Hypothesis 2
As indicated in 5.1.1, the success of the updated hemorrhagic shock and fluid resuscitation model required that we change Hypothesis 2. We had proposed to test Hypothesis 2 using 90 mice with an additional 10 mice for attrition (10%). However, with the aforementioned femoral artery catheterization issues and mouse cage fighting, we were only able to allocate 77 mice instead of 90 mice for Hypothesis 2 testing.

5.1.3 Single Housing
During Year 02, 11 mice sustained wounds from fighting while being group housed. Three of these mice were treated and recovered for testing. We have obtained UNLV IACUC and ACURO approval to house each mouse individually upon arrival to avoid attrition due to fighting.

5.1.4 Hypothesis 2 Flow Cytometry Analysis
We had planned to detect and analyze T and B cell populations in the blood and muscle via flow cytometry. However, as stated in 3.2.2.3.2.2.4.1, we had to omit this detection because of time constraints.

5.1.5 Crush Muscle Injury Practice
The plan was to test 10 mice (Refinement Model Group) to complete Subtask 3: set up of hemorrhagic shock and fluid resuscitation model. We had started this task when the Millar Mikro-Tip® catheter broke (three mice had been used, later designated as Refinement Model Group A Mice). Because of this failure, we were unable to test the rest of the seven mice planned for this experiment that had already been individually housed. Without a functional catheter, these mice would be individually housed longer than we had planned and could not be used. We immediately sought amendment approval from the UNLV IACUC and ACURO to use these mice for inexperienced staff to practice the crush muscle injury procedure and discussed this matter with the Science Officer, Dr. Jennie Conroy, on 1-November-2017. Dr. Conroy approved our corrective action, and these seven mice were referred to as Refinement Model Group B Mice.

5.2 Changes in Personnel
Changes in personnel were as follows:
5.2.1 Resignation of one student worker, Hananeh Derkhshan (August-2017)
5.2.2 Hiring and graduation of the two graduate assistants, Anish Puri and Elizabeth Duffy, and one student worker, April Fisher
5.2.3 Resignation of one student worker, Theofania Mavrantonis (31-October-2017)
5.2.4 Hiring and resignation of one postdoctoral scholar, Dr. Kelley Hammond (07-August-2017 through 30-June-2018)
5.2.5 Hiring of student worker, Jazmin Lopez (04-December-2017)
5.2.6 Hiring of student worker, Karina Statkevich (11-December-2017)
5.2.7 Hiring of engineering consultant, John Parker (01-January-2018)
5.2.8 Hiring of Dr. Andrew Murtishaw temporarily (to replace Dr. Hammond, 18-June-2018 through 15-November-2018)
5.2.9 Hiring of student worker, Jayla Olson (25-June-2018)

5.3 Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them
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.

5.3.1 In the context of expanding our scientific knowledge regarding the effects of fluid resuscitation after hemorrhagic shock and muscle injury, we have generated H2B flow cytometry data that exhibit trends that need to be resolved. Part of the reason that our proposed sample size cannot resolve these trends is that we had a shortfall of 23 mice for Hypothesis 2. This shortfall is due in part to the refinement of the hemorrhagic shock and fluid resuscitation model (see 5.1.2) and the H2B 13% attrition rate from one failed catheterization, early death, or comorbidities during Hypothesis 2 testing. Our plan is to discuss these trends and a plan to test more mice to resolve these trends with the science officer.

5.3.2 The strategies planned for Year 03 to address the overall project delay are as follows:
5.3.2.1 Assign a second staff person to perform femoral arterial catheterization and hemorrhagic shock and fluid resuscitations procedures.
5.3.2.2 Purchase a second set of supplies needed for 5.3.2.1 strategy.

5.3.2.3 Hire a new postdoctoral scholar to complete H2B tissue analysis and maintain a timely Hypothesis 3 tissue analysis.
5.3.2.4 Hire a research technician to assist with animal procedures.
5.3.2.5 Cross-train personnel as much as possible.
5.3.2.6 Complete 50% of 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 02 expenditures are 6 months delayed.

5.5 Significant Changes in Use or Care of Vertebrate Animals
Table 9 lists the Year 02 amendments by the UNLV IACUC-designated package number and provides a succinct description of each amendment. The amendment number represents a continuation from Year 01.

Table 9. Year 02 Animal Protocol Amendments and Approval Dates

UNLV IACUC-Designated Package Number	UNLV Amendment Number	UNLV IACUC Approval Date	ACURO Amendment Number	ACURO Approval Date	Brief Amendment Description
834441-9	5	21-July-2017	5	09-Aug-2017	Personnel; Changes to animal procedures
834441-10	6	07-Aug-2017	6	15-Sep-2017	Addition of “Supplement 6” to ensure clarity of expiration dates of drugs, diluted drugs, stock solutions, and fluids
834441-12[a]	7	31-Aug-2017	7	15-Sep-2017	Personnel; Changes to animal procedures, including, the plantarflexor muscle function testing procedure; Submission of a letter of Dr. Jessica McMorris Muniga’s Millar Mikro-Tip® catheter insertion training at UCLA.

834441-14[b]	8	02-Nov-2017	8	6-Nov-2017	Personnel; Mouse regrouping of due to defective pressure-sensing catheter; Procedure clarifications; Supplement 6 addition (aliquots of fluids)
834441-15	9	27-Nov-2017	9	15-Dec-2017	Single housing days to weeks before procedures
834441-16	10	8-Jan-2018	10	11-Jan-2018	Personnel; Addition of 40 mice; Reallocation of mice; ECG Addition
834441-17	11	5-Mar-2018	11	13-Mar-2018	Personnel; Removal of 10 mice; Revision of hemorrhagic shock, fluid resuscitation, and muscle function testing procedures; Reorganization of Hypothesis 2 into Hypothesis 2A and 2B
834441-19[c]	12	7-June-2018	12	20-June-2018	Personnel; Addition of 60 mice; Changes to hemorrhagic shock and fluid resuscitation procedures

[a]834441-11 was the results of a self-audit and revealed a protocol deviation. This self-audit was reported to ACURO on 31-August-2018.
[b]834441-13 was the annual UNLV IACUC continuing review/progress report. This report was submitted to ACURO on 04-December-2018.
[c]834441-18 was submitted but tabled by the UNLV IACUC for revisions.

6.0 PRODUCTS

6.1 Publications, Conference Papers, and Presentations

Nothing to report

6.2 Journal Publications
Nothing to report

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 10 lists the key individuals who have worked on this project.

Table 10. Project Team Members

Name	**Dr. Barbara St. Pierre Schneider**
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, and performed muscle sectioning when Dr. Hammond resigned.
Funding Support	Not applicable
Name	**Dr. Jessica Muniga**
Project Role	Research Veterinarian
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Assisted with animal protocol amendments, performed femoral

	artery catheterizations, supervised one student worker and one graduate student, and co-handled animal-related tasks.
Funding Support	Not applicable
Name	**Dr. Zhuowei Li**
Project Role	Research Technician
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Assisted with animal protocol amendments, performed crush muscle injury procedure, performed plantarflexor muscle function testing, handled the recording of arterial pressure and heart rate data, and co-handled animal related tasks. Also co-handled animal-related tasks.
Funding Support	Not applicable
Name	**Dr. Liyuan (Angi) Zhang**
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
Name	**Daniela Rincon Cornejo**
Project Role	Project Coordinator
Researcher Identifier	Not applicable
Nearest person month worked	12
Contribution to Project	Managed the grant budget, ordered and tracked laboratory equipment and supplies, and assisted with project planning.
Funding Support	Not applicable
Name	**Dr. Kelley Hammond**
Project Role	Postdoctoral Scholar
Researcher Identifier	Not applicable
Nearest person month worked	11
Contribution to Project	Performed animal and tissue analysis procedures and managed one graduate assistant and two student workers.
Name	**Anish Puri**
Project Role	Graduate Assistant
Researcher Identifier	Not applicable
Nearest person month	4.5

worked	
Contribution to Project	Assisted with animal procedures.
Name	**Elizabeth Duffy**
Project Role	Graduate Assistant
Researcher Identifier	Not applicable
Nearest person month worked	4.5
Contribution to Project	Assisted Dr. Hammond with tissue analysis procedures.
Name	**April Fisher**
Project Role	Student hourly worker
Researcher Identifier	Not applicable
Nearest person month worked	0.6
Contribution to Project	Assisted Dr. Hammond with tissue analysis procedures.
Name	**Jazmin Lopez**
Project Role	Student hourly worker
Researcher Identifier	Not applicable
Nearest person month worked	4
Contribution to Project	Assisted with tissue analysis procedures.
Name	**John Parker**
Project Role	Project Scientist
Researcher Identifier	Not applicable
Nearest person month worked	0.7
Contribution to Project	Assisted with setting up the hemorrhagic shock/fluid resuscitation model.
Name	**Jayla Olson**
Project Role	Student Worker
Researcher Identifier	Not applicable
Nearest person month worked	0.4
Contribution to Project	Assisted with tissue analysis procedures.
Name	**Dr. Andrew Murtishaw**
Project Role	Researcher
Researcher Identifier	Not applicable
Nearest person month worked	1
Contribution to Project	Assisted with tissue analysis procedures.

Name	**Olufunke Gbadamosi**
Project Role	Student Worker
Researcher Identifier	Not applicable
Nearest person month worked	0.5
Contribution to Project	Assisted with tissue analysis procedures.

7.2 Change in the Active Other Support of the PD/PI(s) or Senior/Key Personnel
On 30-June-2018, Dr. Kelley Hammond resigned from the project.

7.3 Research Partners
Table 11 lists the organizations that have served as research partners.

Table 11. Research Partners

Organization Name	**University of Cincinnati**
Location of Organization	Cincinnati, Ohio
Partner’s contribution to the project	Collaboration – review of flow cytometry results

Organization Name	**University of Central Florida**
Location of Organization	Orlando, Florida
Partner’s contribution to the project	Collaboration – statistical analysis

8.0 SPECIAL REPORTING REQUIREMENTS

8.1 Collaborative Awards
Not applicable

8.2 Quad Chart

Veterans Health Administration R&D www.research.va.gov

The Effect of Hypobaria on Muscle Inflammation and Regeneration after Injury and Hemorrhagic Shock

JW150007, Joint Warfighter Medical Research Program

Defense Health Agency

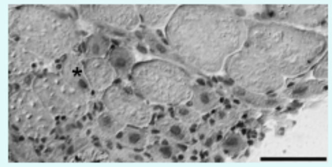
PI: Dr. Barbara St. Pierre Schneider **Organization:** University of Nevada, Las Vegas **Award Amount: $** $5,558,801.00

Study Aim

The overall study aim is to examine the effect of hypobaria on muscle inflammation and regeneration after injury and hemorrhagic shock with fluid resuscitation.

Approach

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.



Regenerating fibers (*) in crush-injured quadriceps muscle (bar = 100 microns)

Accomplishments: We have a working system for measuring arterial blood pressure, withdrawing blood, and instilling fluid. We also have a 90% success rate in inserting femoral artery catheters.

Timeline and Cost

Activities	17	18	19	20
Animal Approval & Hiring				
Hypothesis 1 Testing				
Hypothesis 2 Testing				
Hypothesis 3 Testing				
Estimated Budget	$1,271,577	$1,406,671	$1,447,661	$1,432,892

Goals/Milestones: CY17 and 18 Goals

- ☒ Animal approval, hiring personnel, and model refinement
- ☒ Examine effect of 16 hours of hypobaria on uninjured muscle.
- ☒ Set up hemorrhagic shock model.
- ☐ Examine inflammation activation of fluid resuscitation.
- ☐ **CY18 Goal** – Effect of hypobaria after injury
- ☐ Investigate effect of hypobaria on crush muscle injury and hemorrhagic shock in 190 subjects.
- ☐ **CY19 Goal** – Effect of hypobaria after injury
- ☐ Investigate effect of hypobaria on crush muscle injury and hemorrhagic shock in 215 subjects.
- ☐ **CY20 Goal** – Effect of hypobaria after injury
- ☐ Investigate effect of hypobaria on crush muscle injury and hemorrhagic shock in 123 subjects.
- ☐ **Comments/Challenges/Issues/Concerns**
  - Delays in animal approval, personnel hiring, and setting up hemorrhagic shock/fluid resuscitation model
- ☐ **Budget Expenditure to Date**
- ☐ Projected Expenditure: $5,558,801
- ☐ Actual Expenditure: $1,497,723.75

Updated: 08-Aug-2018

9.0 APPENDICES

Appendix A

Appendix A

The Effect of Hypobaria on Muscle Inflammation and Regeneration After Injury and Hemorrhagic Shock: Hypothesis 1 Report JW150007

Barbara St. Pierre Schneider, PhD, RN, CNE

Principal Investigator

26 September 2017

Table of Contents

Technical Abstract

Currently, U.S. military en route care consists of an 8–9 flight period, transporting wounded warfighters from the Middle East to Europe. This flight period may be expanded to 16 hours (hr) if the Pacific theater develops into a more active region for military conflict in the future. Therefore, research examining the effect of an extended time period of hypobaria is needed to ensure there are no adverse tissue effects associated with such care or to identify appropriate countermeasures, if necessary. The primary objective of this study was to test the effect of 16-hr of hypobaria on overall physiologic functioning, skeletal muscle morphology, and resident leukocyte populations in mice. The study hypothesis was overall well-being, general muscle morphology, and muscle leukocyte populations will be similar between male mice exposed to hypobaria for 16 hr and male mice exposed to normobaria. Mice were assigned to a normobaric (NB) or hypobaric (HB) chamber for 14–17 hr and then euthanized. Major findings were as follows: (a) mice behaved normally during 14–17 hr of hypobaria; and (b) the number of CD206- and CD68-positive cells in three muscle types was similar between NB and HB mice. One unexpected finding was that the lateral aspect of the quadriceps femoris muscle consisted of approximately 33% more CD206-positive cells than the lateral gastrocnemius ($p < .05$). Collectively, these findings contribute to our understanding of the effects of 14–17 hr of hypobaria and may contribute to the scientific foundation for treating wounded troops during en route care.

Keywords: hypobaria; lateral gastrocnemius; leukocytes; quadriceps femoris; skeletal muscle

Introduction

The U.S. military currently provides overseas en route care for wounded soldiers and soldiers with medical conditions. Typical en route care covers an 8–9-hr flight period and contributes to a combat survival rate up to 99% [1].

Despite its contribution to a high survival rate, en route care includes challenges. For example, the fixed wing aircraft typically used for overseas en route care has a cabin atmospheric pressure equivalent to that of an altitude of 8,000 ft. At this altitude, oxygen content is lower than that of sea level, and therefore, the possibility exists that the environmental oxygen supply may not be adequate for certain transported wounded individuals. For example, during a 22-month period, 55 of 61 casualties who were flown from Bagram Air Base in Iraq to Landstuhl Regional Medical Center (mean transport time was 9.3 hr) experienced at least one hypoxemic episode—defined as a recorded oxygen saturation < 90% [2]. For these individuals, the mean hypoxemic duration was 40 min [2]. Furthermore, for approximately 12 min, 56% of these patients exhibited oxygen saturation < 85% [2]. Although the outcome of these hypoxemic events was not studied collectively, these findings suggest that the airplane's environmental oxygen supply may be insufficient for proper tissue oxygenation. Insufficient tissue oxygenation could exacerbate existing injuries.

In a previous report, the effect of the typical en route care time, 8–9 hr, and aircraft cabin pressure—565 torr—on skeletal muscle resident leukocyte populations and gene expression was studied [3]. Although this single exposure of hypobaria led to the activation of certain epithelial-related genes or microRNAs in healthy skeletal muscle of female and male mice, respectively; hypobaria did not activate pro-inflammatory genes or leukocytes [3].

The possibility exists that en route care may be expanded to a 16-hr flight period if the Pacific theater develops into a more active region for military conflict in the future. Therefore, research examining the effect of an extended time period of hypobaria is needed to ensure there are no adverse tissue effects associated with such care or to identify appropriate countermeasures, if necessary. The primary objective of this study was to test the effect of 16-hr of hypobaria on overall physiologic functioning, skeletal muscle morphology, and resident leukocyte populations in mice. The study hypothesis was overall well-being, general muscle morphology, and muscle leukocyte populations will be similar between male mice exposed to hypobaria for 16 hr and male mice exposed to normobaria.

Methods

Animals

All animal procedures were reviewed and approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee and the U.S. Army Medical Research and Materiel Command Animal Care and Use Review Office. Male (n = 22) C57BL/6 mice (*Mus musculus*; approximately 6-weeks old and 20 g) were purchased from Envigo Laboratories (Indianapolis, IN) and allowed at least 5 days to acclimate before the start of the study. Animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at the University of Nevada, Las Vegas. Mice were group housed (4–5 per ventilated cage [Lab Products; Seaford, DE] with ¼” corn-cob bedding [The Andersons/Bed-O’Cobs; Maumee, OH]) under a 12:12-h light:dark cycle. Cotton nesting material was provided for enrichment (Nestlets; Ancare; Bellmore, NY). Tap water and rodent chow (Lab Diet 5001; Purina Mills Inc.; St. Louis, MO) were available ad libitum.

Experimental Design

Figure 1 shows the experimental design. Mice were evenly assigned to exposure to hypobaria (target 565 ± 5 torr) or normobaria for 14−17 hr. Each exposure occurred in an acrylic chamber as described previously [3]. Body weight was measured before placement within the chamber. Each mouse entered a chamber with an atmospheric pressure > 700 torr. Thirty minutes after chamber placement and each hour thereafter, each mouse was visually monitored for quality and depth of respirations, mouth color, posture, and activity level. Chamber pressure and CO_2 level were also measured at the same time points [3]. At the end of the exposure period, each mouse was removed from the chamber and weighed. All 22 mice were tested within one month upon arrival.

Euthanasia and Tissue Harvest

After postchamber weighing, each mouse was anesthetized with inhalant isoflurane (1–5%) and 100% oxygen. Blood was collected from an axillary blood vessel, immediately stored at 0–4°C to clot, centrifuged (Eppendorf 5417c; Hamburg, Germany) for 10 min at 1,882 × *g*, and then stored at –70°C.

After blood collection, each anesthetized mouse was euthanized by cervical dislocation, and then the plantarflexor and quadriceps femoris (QF) muscles were harvested bilaterally and frozen in melting isopentane (Fisher Scientific; Pittsburgh, PA) cooled by liquid nitrogen. All frozen muscles for leukocyte analysis were then stored at −70°C. In addition, the spleen was harvested and weighed posteuthanasia to determine whether splenomegaly occurred after HB exposure.

Preparation of Muscle Cross-Sections for Leukocyte Analysis

Ten-micron thick serial cross-sections at or near the midbelly of the gastrocnemius and QF muscles were cut using a cryostat (Leica CM1850; Leica Microsystems Inc.; Bannockburn, IL). Cross-sections were applied to positively-charged or poly-L-lysine-coated slides and stored at –70°C until immunolabeling. In addition, cross-sections were fixed in methanol and stained with hematoxylin and eosin (H&E) for morphological analysis.

Leukocyte Analysis in Muscle Region of Interest

Immunolabeling. To detect the presence of leukocytes in frozen muscle cross-sections, immunolabeling was used—following a similar procedure as described previously [4]. The primary monoclonal antibodies were: (a) rat anti-mouse CD206 (MR5D3; 1:100; Bio-Rad; Hercules, CA); (b) rat anti-mouse CD68 (FA-11; 1:100; Bio-Rad); (c) rabbit anti-mouse CD163 (EPR19518; 1:20; Abcam; Cambridge, MA); and (d) rat anti-mouse F4/80 (BM8; 1:20; Invitrogen; Frederick, MD). These antibodies detect proteins present in macrophages [5-7]. In addition, control sections were generated as described above except the primary antibody step was substituted by immersing the sections in phosphate-buffered saline for 2 hr.

Image Analysis. Of the four antibodies, the anti-CD206 and CD68 antibodies yielded consistent and significant immunolabeling. That is, at least 70% of the mice within each pressure condition had at least three medium-intense-immunolabeled cells. Only anti-CD206 and CD68-antibody-immunolabeled and control sections underwent image analysis.

One image of the region of interest (ROI; identified as an area with the most cells) within one cross-section of the lateral gastrocnemius (LG), medial gastrocnemius (MG), and QF muscles was captured using a 10x objective (100x magnification) attached to an Eclipse E600 light microscope (Nikon Inc.; Melville, NY), a Retiga 2000R CCD Camera (QImaging, Surrey, BC; Canada), and Image-Pro® Premier 9.2 software (Media Cybernetics Inc.; Silver Spring, MD). Point background correction was performed by Image-Pro® Premier 9.2 software. Each image represented one ROI. A total of 42 LG, 21 MG, and 22 QF images were captured. In addition, an image from control sections was matched with the corresponding immunolabeled section.

After image capturing, the fibers located along the edge of the immunolabeled images were matched to those in the control section, and any unmatched areas in CD206 or CD68 ROIs were removed from analysis. Debris in CD206 or CD68 ROIs were labeled for pre-processing and excluded (see next paragraph). The range of the adjusted ROI areas was 0.70–1.02 mm^2.

Automated quantification of the immunolabeled images was performed using MATLAB software (MATLAB and Statistics Toolbox Release 2016a; The MathWorks Inc.; Natick, MA). The detailed procedure has been described by Jiao *et al.* [8]. First, pre-processing occurred with the conversion of the input image of 1600 x 1200 pixels to gray scale and the calculation of a global mean intensity value to correct image artifacts. The input image was then divided into blocks of 400 x 400 pixels, and the local mean intensity value and the standard deviation σ were calculated.

The second step was image segmentation. For each block, the local threshold was obtained by applying the localized (LI) iterative Otsu's threshold method. In addition, traditional Neural Networks and Convolutional Neural Networks classifiers were adopted to separately classify CD206- and CD68-positive cells. The results were merged with those generated from LI Otsu's method to reduce error rate.

The third step, muscle cross-section edge detection, was performed in two substeps. The first substep involved detecting the muscle texture when the periphery of the muscle cross-section was present in the image. Then the fuzzy detection method of leukocytes was performed because sometimes leukocytes accumulated near the periphery of the muscle (e.g., near the epimysium). Finally, the muscle cross-section edge was determined by merging the results of the two substeps.

The last step of the framework was ROI selection and block-based analysis. The ROI selection was based on the block unit with the block size of 200 x 200 pixels. A block with more than 50% of "empty" space was excluded from the ROI. The objects within each block that were considered as part of the ROI and analyzed were those at a distance greater than 100 pixels from the muscle cross-section edge.

The variables quantified for the LG, MG, and QF muscles were the number of CD206-positive cells/mm^2 or CD206 number/mm^2, the mean size of CD206-positive cells/ROI or mean CD206 antigen area, and the total ROI area positive for CD206 or CD206 area percentage. For the LG only, CD68 number/mm^2, mean CD68 antigen area, and CD68 area percentage were determined. Means, standard deviations (SD), and medians were calculated for the NB and HB groups and used to test the study hypotheses.

Statistical Analyses

For all statistical analyses, the confidence level and alpha were set at 95% and 5%, respectively. Outcomes were treated independently. When comparing the means, the normality assumption was checked using the Q-Q plots and then the Folded test for equality of variances was performed. If the assumption of equal variances was supported, then the pooled variance estimation approach was used. Otherwise, the Satterthwaite method was used. A 95% confidence interval based on the *t*-distribution was constructed for mean differences. If the confidence interval contained zero, then we concluded no significant difference between the means. The medians of the (a) absolute differences of body weight before (BW1) and after (BW2) exposure to normobaria or hypobaria and (b) rates of change in body weight |BW1-BW2|/BW1 were analyzed by Wilcoxon signed-rank test. Repeated measure analysis of variance with Greenhouse-Geisser correction was performed to determine the relationship between chamber pressure and time, and chamber CO_2 levels and time.

RESULTS

Chamber Pressure and CO_2 Levels

During the experiment, the NB chamber pressure ranged from 700–726 torr and the HB chamber pressure ranged from 563–570 torr. During the 14–17-hr chamber exposure, mean pressure remained stable in both the NB and HB chambers ($p = 0.31$). However, the mean pressure was lower in the HB chamber than in the NB chamber ($p < 0.001$).

Across the exposure period, the CO_2 level changes were similar for both NB and HB conditions ($p = 0.06$), with the CO_2 level ranging from 385–659 ppm in the NB chamber and 321–479 ppm in the HB chamber. However, mean CO_2 level in the HB chamber was significantly lower than in the NB chamber ($p < 0.001$).

Mouse Behavior and Body and Spleen Weights

During and after HB and NB exposure, the mice exhibited normal (a) respirations, (b) mouth color, and (c) posture. Piloerection of fur was not observed. While the mice were inside the HB and NB chambers, they behaved normally, such as nesting, sleeping, grooming, eating, and drinking water.

Table 1 shows the results of body weight comparisons. Although the body weight change was only approximately 4%, this change was statistically significant ($p < 0.0001$). However, as indicated, the rate of body weight change was similar for both exposure conditions. Furthermore, the absolute differences of the body weight before and after pressure exposure did not differ significantly between the two conditions.

Mean relative spleen weight was similar between the NB (2.56 ± 0.24 mg/g) and HB (2.42 ± 0.24 mg/g) conditions. The 95% confidence interval is [-0.07, 0.35].

Muscle Morphological Analysis

For both conditions, muscle fibers in LG, MG, and QF muscles exhibited no variation in eosin staining. In addition, nuclei were primarily located peripherally around muscle fibers. Therefore, no evidence of inflammation was observed in the muscles exposed to hypobaria (Figure 2).

Leukocyte Analysis

CD206 immunolabeling. No false-positive immunolabeling was observed in any control sections. CD206-positive cells were primarily observed within the perimysium (Figure 3A, B, and C). Cells varied in shape and fill and exhibited an overall strong immunolabeling intensity (Figure 3D and E). No difference in CD206 number/mm^2 (Figure 3F), CD206 mean antigen area (Figure 3G), and CD206 area percentage was observed between the NB and HB conditions for each muscle. When muscles were compared, the mean LG CD206 number/mm^2 (44.22 ± 15.78, n = 21) of NB and HB mice combined was significantly lower than that of QF (66.06 ± 18.71, n = 22) of NB and HB mice combined ($p < 0.05$, Figure 3H).

CD68 immunolabeling. No false-positive immunolabeling was observed in any control section. The CD68-positive cells were located in the periphery of muscle fibers and had an elongated or roundish shape. For LG muscle, no difference in CD68 number/mm^2 ($p = 0.30$, Figure 3I), mean antigen area ($p = 0.75$), and area percentage ($p = 0.27$) was observed between the NB and HB exposure conditions.

F4/80 and CD163 immunolabeling. The F4/80 protein was absent around muscle fibers in seven LG muscle cross-sections (NB mice, n = 5; HB mice, n = 2). In three QF muscle cross-sections (NB mice, n = 3), the CD163 protein was absent around muscle fibers, but present in thick connective tissue near the vastus lateralis, which is one of the four muscles composing the QF.

DISCUSSION

Our experiments demonstrated a stable, reproducible model to study extended—14–17- hr—HB exposure. During the chamber time, the healthy mice showed normal behavior, rate of respiration, and posture. The morphology of the LG, MG, and QF muscles was normal. In addition, the presence of CD206-positive and CD68-positive cells in hindlimb muscle is similar between the NB and HB conditions. Our results supported the study hypothesis.

Our findings of normal mouse well-being, normal general muscle morphology, and no difference in muscle resident CD68-positive cells of mice exposed to 14–17-hr is similar to our previous findings in mice exposed to 8–9 hr of hypobaria [3]. Therefore, extended exposure to an atmospheric pressure equivalent to that in a military transport airplane appears to have no major effect on overall well-being, general muscle morphology, and muscle resident leukocyte populations.

One unexpected finding in the current study is the presence of CD206-positive cells in healthy muscles of both NB and HB mice. The protein, CD206, is a marker of M2 macrophages, and these macrophages are involved in tissue-remodeling processes [7]. Specifically, M2 macrophages enhance myoblast differentiation and fusion and increase collagen production by fibroblasts cell culture [9]. Although our findings do not indicate the function of CD206-positive cells in muscle that is not injured or undergoing remodeling, the possibility exists that these cells may be present in normal muscle because of the role of the CD206 protein in collagen turnover [10], and collagen is present throughout muscle.

In conclusion, extended exposure of hypobaria (~565 torr) has no major effect on healthy tissue, suggesting a 16-hr flight alone may have no major influence on healthy soldiers or passengers. The next step is to determine whether this extended exposure of hypobaria will affect recovery after crush muscle injury and hemorrhagic shock.

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Table 1. Body Weight Summary Statistics

Condition[a]	Mean ± SD	Median	*t (df)*	95% Confidence Interval	*p*-Value
NB					
BW1	20.58 ± 1.15	20.40			
BW2	21.54 ± 1.18	21.20			
BW1–BW2	-0.96 ± 0.50		-6.32 (10)	[-1.30, -0.62]	< 0.0001
HB					
BW1	21.13 ± 0.95	21.00			
BW2	21.97 ± 1.00	22.22			
BW1–BW2	-0.84 ± 0.42		-6.69 (10)	[-1.12, -0.56]	< 0.0001

[a]*n* = 11 per exposure group; NB = normobaric; HB = hypobaric; BW1 = body weight before the exposure; BW2 = body weight after the exposure

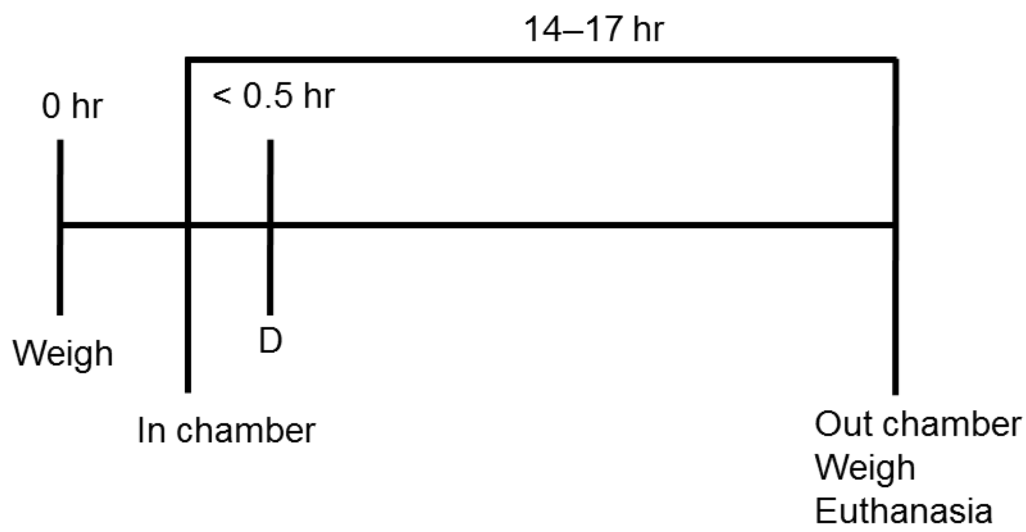

Figure 1. Experimental Design. After weighing, each mouse was placed inside an NB or HB chamber. Each mouse was observed every 60 min ± 15 min for 14–17 hr. Then the mouse was removed from the chamber, weighed, and euthanized. NB = normobaric; HB = hypobaric; D = depressurization

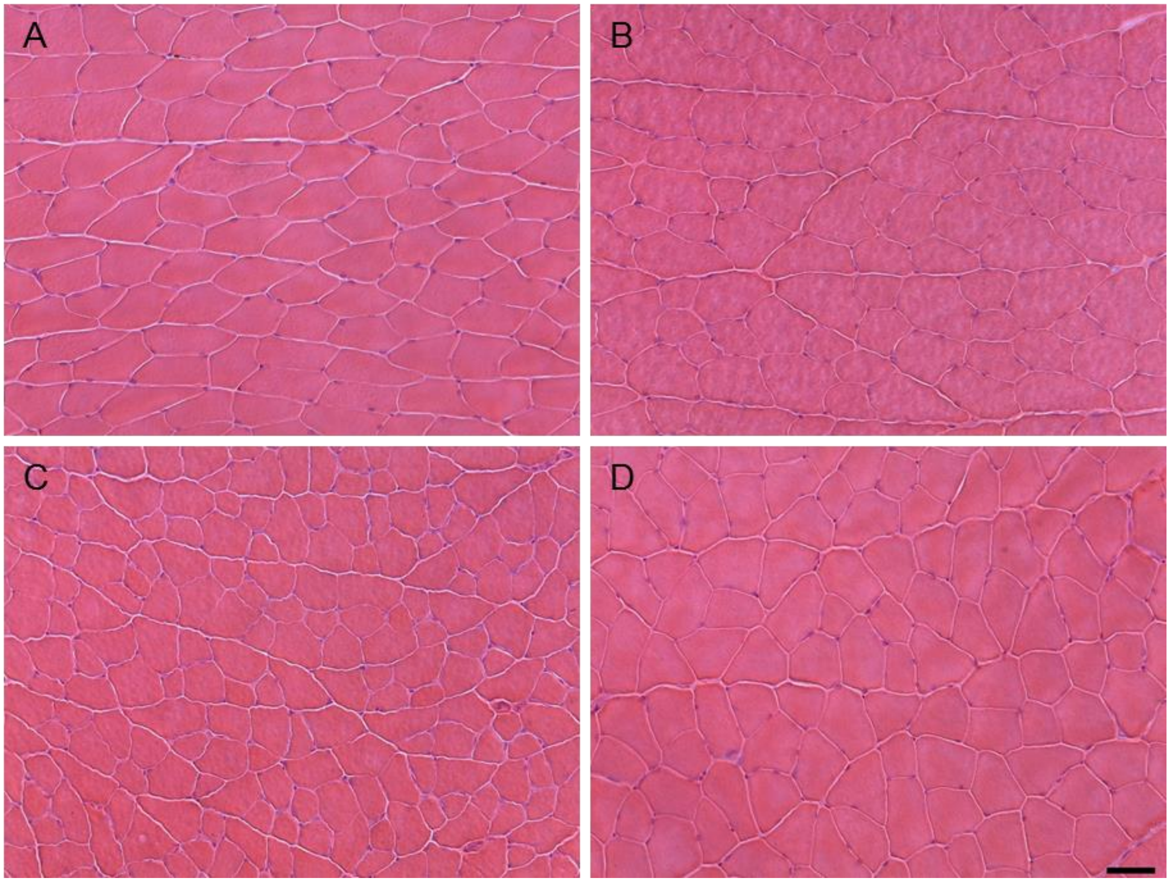

Figure 2. Morphology of LG and QF Muscle Cross-sections of Mice Exposed to NB and HB Conditions. The LG of NB (A) and HB (B) mice exhibits normal morphology. The QF of NB (C) and HB (D) mice exhibits normal morphology as well. Hematoxylin and eosin, bar = 50 µm. LG = lateral gastrocnemius; QF = quadriceps femoris; NB = normobaric; HB = hypobaric

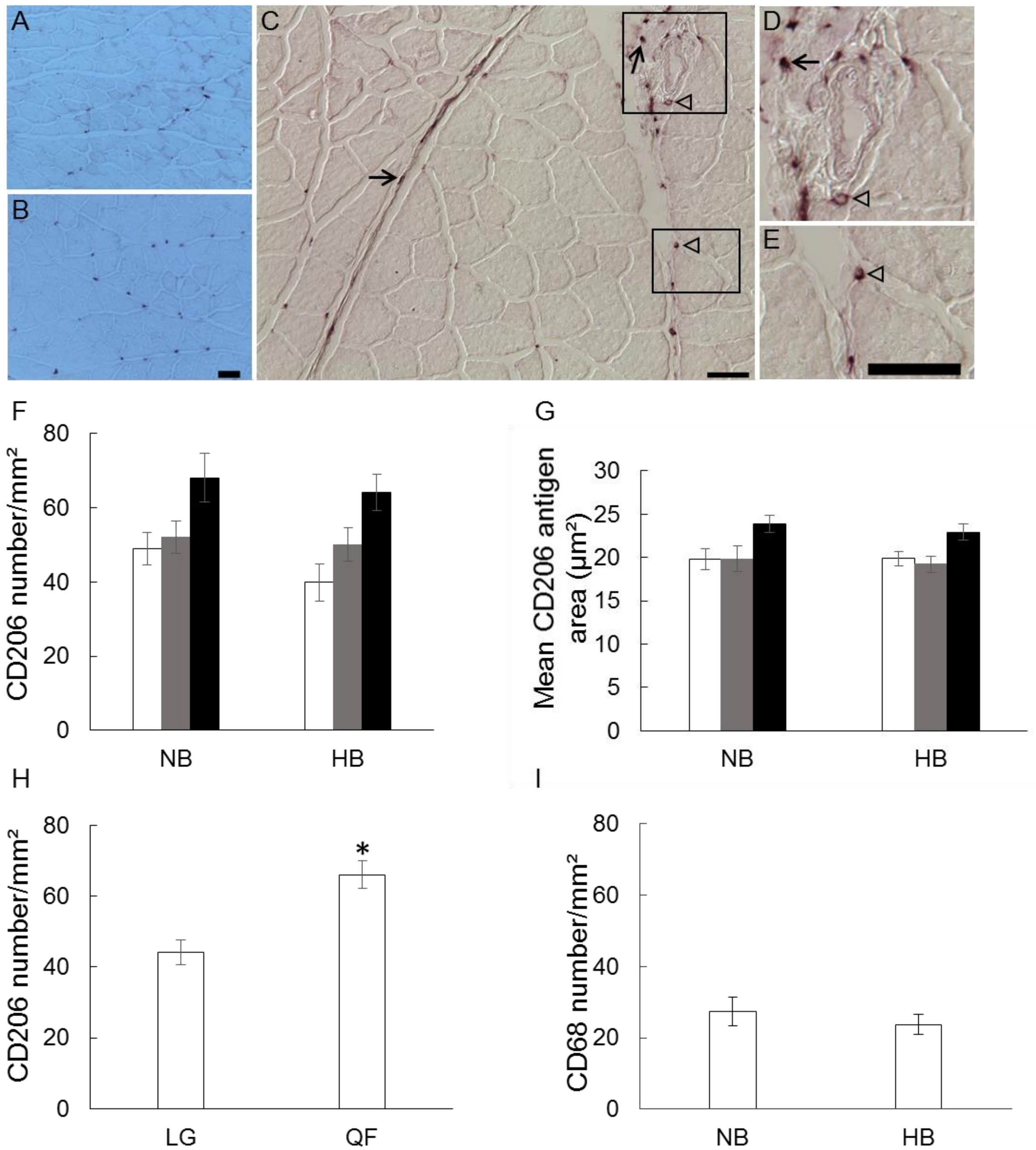

Figure 3. Leukocyte CD206 and CD68 Immunolabeling of LG, MG, and QF Muscle Cross-sections of NB and HB Mice. CD206-positive cells are present within LG (A) and QF (B) cross-sections (50 µm bar) of a HB mouse. (C) In this image, which was captured from the QF of a HB mouse, the shape and fill of CD206-positive cells varies. Both roundish and elongated (arrows) cells are present as well as completely (arrows in C and D) and partially filled cells (arrowheads in C–E). The overall intensity of the immunolabeling is strong. Bar = 50 µm. Mean CD206 number/mm^2 (F) and mean CD206 antigen area (G) are similar between the NB (n = 11) and HB (n = 11) conditions. (H) Mean CD206 number/mm^2 in the LG of NB and HB mice combined (n = 21) is significantly lower than that of QF of NB and HB mice combined (n = 22, $p < 0.05$). (I) Mean LG CD68 number/mm^2 does not differ between the

NB (n = 11) and HB (n = 11) conditions. NB = normobaric; HB = hypobaric; □ LG = lateral gastrocnemius; ■ MG = medial gastrocnemius; ■ QF = quadriceps femoris; Values are means ± standard error.