NEW INSIGHTS INTO POST-SEPSIS MUSCLE WEAKNESS ELUCIDATED USING A NOVEL ANIMAL MODEL

Allison M. Steele

University of Kentucky, a.steele@uky.edu

Author ORCID Identifier: https://orcid.org/0000-0001-5623-4891

Digital Object Identifier: https://doi.org/10.13023/ETD.2017.476

Click here to let us know how access to this document benefits you.
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Allison M. Steele, Student
Dr. Hiroshi Saito, Major Professor
Dr. Kenneth S. Campbell, Director of Graduate Studies
NEW INSIGHTS INTO POST-SEPSIS MUSCLE WEAKNESS ELUCIDATED USING A NOVEL ANIMAL MODEL

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Allison Marie Steele

Lexington, Kentucky

Co-Directors: Dr. Hiroshi Saito, Professor of Surgery and Dr. Francisco Andrade, Professor of Physiology

Lexington, Kentucky

2017

Copyright © Allison M. Steele 2017
NEW INSIGHTS INTO POST-SEPSIS MUSCLE WEAKNESS ELUCIDATED USING A NOVEL ANIMAL MODEL

Sepsis is a severe life-threatening critical illness that damages multiple physiological systems. After hospital discharge, more than 70% of severe sepsis survivors report profound weakness which significantly impacts quality of life. Such weakness gives rise to new limitations of daily living, which ultimately leads to loss of independence in many patients. Despite wide recognition of this serious issue by clinicians and researchers alike, the mechanisms contributing to chronic skeletal muscle dysfunction after sepsis are not well understood. Lack of progress in this field is largely due to the absence of an appropriate animal model; current models are either too mild to induce muscle weakness or too severe and cause death within a few days. As such, this dissertation work first focused on establishing a clinically-relevant animal model of sepsis which yields surviving mice with chronic skeletal muscle weakness (Aim 1). This aim involved refining the cecal slurry injection model of polymicrobial sepsis in young adult animals, as well as optimizing the timing, duration, and dose of multiple therapeutic agents. The resulting resuscitation protocol was adapted for use in late-middle-aged animals, and muscle strength was evaluated using an \textit{ex vivo} system which confirmed significant muscle weakness in sepsis survivors, long after sepsis was resolved. Next, using this novel model, we sought to characterize sepsis-induced long-term muscle dysfunction at the molecular level (Aim 2). The first set of experiments under this aim was designed to identify the primary global mechanism(s) (i.e. atrophy, polyneuropathy, and/or myopathy) responsible for muscle weakness in sepsis survivors. Analysis of the force-frequency curves and specific force measurements led to the conclusion that myopathy is the primary cause. Electron micrograph observation, functional assays, and protein analysis then showed that sepsis survivors' skeletal muscles are characterized by profound mitochondrial abnormalities and oxidative damage. Collectively, these studies demonstrate that long-term muscle weakness is apparent in sepsis-surviving animals, and the functional decline is associated with unresolved mitochondrial damage and dysfunction. This work
suggests that medical treatments beyond targeting muscle wasting alone could allow sepsis survivors to regain function and return to productive lives.

KEYWORDS: Mitochondria, Muscle weakness, Myopathy, Oxidative damage, Sepsis
NEW INSIGHTS INTO POST-SEPSIS MUSCLE WEAKNESS ELUCIDATED
USING A NOVEL ANIMAL MODEL

By

Allison M. Steele

Dr. Hiroshi Saito
Co-Director of Dissertation

Dr. Francisco H. Andrade
Co-Director of Dissertation

Dr. Kenneth S. Campbell
Director of Graduate Studies

November 22, 2017
To my grandmother,

As you taught me how to plant seeds in a flower bed,
You also planted seeds in my young mind;
Seeds of ideas of a promising future,
And the principle of taking responsibility of my own dreams.
It is with your wisdom that my life has grown to what it is today,
And I will forever be your “wormie.”
ACKNOWLEDGEMENTS

It is with sincere gratitude that I thank my mentor, Dr. Hiroshi Saito, who has instilled in me a strong sense of scientific curiosity and perseverance while I have been fortunate enough to be his graduate student. Your mentorship has also translated to my personal growth, for which I am equally thankful.

Next, I thank committee member Dr. Marlene Starr, who was a post-doctoral fellow in the laboratory when I first joined. Your daily support has been a key driver in getting me to this point today. Thank you for being my teacher, my mentor, and for leading by example.

I am highly appreciative of my co-mentor, Dr. Francisco Andrade; thank you for challenging me to think critically, and for your help in framing my data into a story. I would like to express my thanks to members of my advisory committee, Drs. Karin High, Subbarao Bondada, Samir Patel, and former member Dr. Karyn Esser. Your guidance has helped me develop critical thinking, taught me to prioritize, and how to focus my attention; thank you for your dedication and involvement in my training.

A great thank-you to the Department of Physiology for providing an education and sense of community for which I am extremely fortunate and grateful. I would like to specifically thank our Director of Graduate Studies, Dr. Ken Campbell, for your dedication to us PGY students collectively and individually. Additionally, I’d like to thank the Center for Muscle Biology, and its director and co-sponsor of my fellowship, Dr. Charlotte Peterson; thank you for expanding my knowledge of muscle biology and providing resources to learn and perform crucial techniques for this project. Further, thank you to the Department of Surgery, Markey Cancer Center, and CCTS at the University of Kentucky, and the Harvard Medical School Electron Microscopy Facility, which have all largely contributed to my project.

My educational endeavor, however, would likely be unexplored or unfinished without the support of my family. I would like to thank my “step” mother, Laurie, for having confidence in my abilities when I did not, and encouraging me to enroll in honors classes in high school, which honestly was the spark of this entire journey. Thank you to my father, Frank, not only for your daily support but also for learning about my project which has meant the world to me. Thank you to my mother for always being willing to give me what I needed even though it was often beyond her personal interest.

A special thank-you to Michael, my better half; you have brought joy and inspiration to my life at a time when I needed it the most. Your support and patience have been limitless, and I hate to say that even your cheesy jokes never failed to make me smile and keep me going when I was otherwise overwhelmed.
Thank you to my “twin” Kendra; you have been with me every step of this winding path of graduate school (and life), and I cannot thank you enough. Thank you, Brittany, for finding ways to support and encourage me, even from a distance. You have been my friend, sister, but also role model and I am so thankful to have you in my life. Also, thank you Beverly not only for your help in lab but also always bringing a positive energy.

Finally, I’d like to thank my undergraduate research mentors, Drs. Sarah Mordan-McCombs and Edward Chikwana, for sparking the research bug in me, seeing my potential, and starting me on this journey. My life has been changed through your confidence and guidance, and I hope that I can ‘pay it forward’ one day.
TABLE OF CONTENTS

Acknowledgements .................................................................................................................. iii
List of Tables .............................................................................................................................. ix
List of Figures ............................................................................................................................ x

1 Chapter 1: Introduction and Hypothesis
  1.1 Sepsis ............................................................................................................................... 1
    1.1.1 Pathogenesis of sepsis ............................................................................................... 1
    1.1.2 Sepsis epidemiology and survival outcomes .............................................................. 3
  1.2 Chronic muscle weakness after sepsis ............................................................................. 4
    1.2.1 Muscle weakness in patients with sepsis during their stay in the ICU ..................... 4
    1.2.2 Persistent muscle weakness and chronic disability in sepsis survivors ...................... 8
  1.3 Lack of an appropriate animal model as limitation of previous studies ......................... 11
  1.4 Significance of using middle-aged animals .................................................................... 14
  1.5 Study rationale .................................................................................................................. 15
    1.5.1 Critical barrier to progress in the field ....................................................................... 15
    1.5.2 Central hypothesis .................................................................................................... 16
    1.5.3 Specific aims ............................................................................................................ 16

2 Chapter 2: Refinement of the Cecal Slurry Model of Experimental Sepsis
  2.1 Abstract ............................................................................................................................ 18
  2.2 Introduction ...................................................................................................................... 19
  2.3 Experimental approach ................................................................................................... 20
  2.4 Results .............................................................................................................................. 21
    2.4.1 Cecum size, shape, and cecal content consistency varies widely among different experimental conditions .............................................................................................................................. 21
    2.4.2 Cecal slurry bacteria remain bioactive after long-term storage when prepared using our refined protocol .................................................................................................................. 26
    2.4.3 CS-induced sepsis reproduces age-dependent sepsis severity and mortality ............ 27
    2.4.4 The severity of CS-induced bacteremia is associated with mortality ......................... 28
    2.4.5 CS-induced death is not mediated by endotoxemia .................................................... 29
  2.5 Discussion ......................................................................................................................... 31
  2.6 Conclusions ...................................................................................................................... 34

3 Chapter 3: Development of a Late-Intervention ICU-Like Resuscitation Model of Sepsis Which Results in High Survival
  3.1 Abstract ............................................................................................................................. 36
  3.2 Introduction ...................................................................................................................... 37
3.3 Experimental approach ................................................................. 38
3.4 Results .......................................................................................... 40
  3.4.1 The majority of animals cannot be rescued by delayed antibiotic treatment .............................................. 40
  3.4.2 Late therapeutic intervention with a combination of antibiotics and fluid resuscitation rescues the majority of animals ................................................. 43
  3.4.3 Delayed, but not early, therapeutic intervention allows for the development of cytokinemia, organ dysfunction, and sustained body weight loss ................................................. 48
3.5 Discussion ...................................................................................... 54
3.6 Conclusions .................................................................................... 58

4 Chapter 4: Characterization of Muscle Weakness in Sepsis Surviving Mice: Muscle Quantity vs. Quality
  4.1 Abstract ......................................................................................... 60
  4.2 Introduction .................................................................................. 61
  4.3 Experimental approach ................................................................. 62
  4.4 Results .......................................................................................... 63
    4.4.1 Animals rescued using our sepsis/resuscitation protocol exhibit chronic muscle weakness ......................... 63
    4.4.2 Behavior-based assessments of rodent strength were not robust enough to make conclusions on the progression of muscle weakness in sepsis survivors ................................................. 69
    4.4.3 Sepsis induces long-term changes in activity ............................................................... 70
    4.4.4 Sepsis-induced atrophy is evident using our sepsis/resuscitation model, and is recovered by day 14 after sepsis induction ............................................................... 73
    4.4.5 Skeletal muscle of sepsis survivors exhibit hallmarks of critical care myopathy ............................................................... 78
  4.5 Discussion ...................................................................................... 80
  4.6 Conclusions .................................................................................... 82

5 Chapter 5: Mitochondrial Myopathy and Oxidative Damage are Associated with Chronic Muscle Weakness in Sepsis Survivors
  5.1 Abstract ......................................................................................... 83
  5.2 Introduction .................................................................................. 84
  5.3 Experimental approach ................................................................. 85
  5.4 Results .......................................................................................... 86
    5.4.1 Skeletal muscle mitochondria of sepsis survivors are characterized by gross structural abnormalities ............................................................... 86
    5.4.2 The integrity of mitochondrial DNA is similar among sepsis survivors and non-sepsis controls ............................................................... 88
    5.4.3 Sepsis induces long-term impairment of mitochondrial function ............................................................... 89
    5.4.4 ATP content is reduced in skeletal muscles of sepsis survivors ............................................................... 93
5.4.5 Oxidative damage accumulates in skeletal muscle of sepsis survivors weeks after initial insult ........................................... 94
5.5 Discussion .................................................................................. 96
5.6 Conclusions ................................................................................ 98

6 Chapter 6: Discussion of Major Findings and Future Directions
6.1 Major findings .............................................................................. 99
6.2 Future directions .......................................................................... 100
   6.2.1 Application of our newly refined protocols to specific and broad research questions .......................................................... 100
   6.2.2 Further characterization of cecal slurry and post-sepsis muscle weakness .................................................................... 101
   6.2.3 Further improvements to model ................................................................................................................................. 105
   6.2.4 Use of transgenic animals to establish causal relationship between mitochondrial myopathy and weakness .................................................. 106
   6.2.5 Elucidation of therapeutics ......................................................................................................................... 108
6.3 Overall summary .......................................................................... 110

7 Chapter 7: Thesis Methodology
7.1 Animals and husbandry ................................................................. 111
7.2 Induction of chronic pancreatitis and diabetes in mice .............. 111
7.3 Cecal slurry stock preparation and characterization ................. 112
7.4 Induction of polymicrobial abdominal sepsis by cecal slurry injection ..................................................................................... 114
7.5 Assessment of bacteremia ............................................................ 115
7.6 Therapeutic intervention ............................................................... 115
   7.6.1 Antibiotics and fluid resuscitation ........................................... 115
   7.6.2 Cage warming ........................................................................ 116
7.7 Analysis of cytokinemia ................................................................. 119
7.8 Evaluation of lung and liver injury .............................................. 119
7.9 Assessments of muscle mass ......................................................... 120
7.10 Muscle strength analysis using an ex vivo system ..................... 121
7.11 Behavioral analyses of strength and activity ............................ 123
7.12 Muscle histochemistry ................................................................. 124
   7.12.1 Tissue processing ................................................................. 124
   7.12.2 Image acquisition ................................................................. 124
   7.12.3 Hematoxylin and Eosin (H&E) staining and quantification ...................................................................................... 124
   7.12.4 ATPase histochemical stain .................................................. 125
   7.12.5 Immunofluorescent fiber-type staining and semi-automated quantification of cross-sectional area .................................. 125
7.12.6 Histochemical staining of electron transport chain complex enzyme activities ................................................................. 126
7.12.6.1 NADH dehydrogenase staining ......................................................... 126
7.12.6.2 SDH staining ................................................................................. 127
7.12.6.3 COX staining ................................................................................. 127
7.12.6.4 Image quantification using Aperio ScanScope software ......................................................................................... 127
7.13 Transmission electron microscopy ............................................................... 127
7.14 Mitochondrial isolation and respiration analysis ............................................. 128
7.15 Analysis of mitochondrial DNA integrity using PCR assay ......................... 130
7.16 ADP/ATP quantification ........................................................................... 132
7.17 Protein isolation and western blot analysis ................................................. 132
7.18 Overall statistical analysis ........................................................................ 133

Appendices
Appendix 1. Abbreviations ............................................................................... 135

References ......................................................................................................... 137

Vita ................................................................................................................... 150
LIST OF TABLES

1.1 Muscle studies using current animal models of sepsis .......................... 12
7.1 Animal cohort stratification by outcome measure with detailed sepsis survival rate .............................................................................................................................. 117
7.2 Final concentration of working Krebs-Ringers solution ............... 122
7.3 PCR conditions ...................................................................................... 131
LIST OF FIGURES

1.1 Progression of sepsis pathogenesis .......................................................... 2
1.2 The number of sepsis survivors in the US has doubled within the past decade .......................................................... 4
1.3 Increased sepsis severity is associated with more profound reductions in health-related quality of life. ...................................... 10
1.4 The lack of an appropriate animal model of sepsis has been a critical barrier to evaluating mechanisms of long-term muscle weakness in sepsis survivors .......................................................... 11
1.5 Risk of sepsis incidence and sepsis-mediated mortality increase with advancing age .......................................................... 15
2.1 Abnormal consistency of cecal contents in diabetic mice ...................... 22
2.2 Mice with chronic pancreatitis have enlarged ceca ............................. 23
2.3 Cecal shape is highly variable in aged mice ........................................ 24
2.4 Cecal bacterial flora are similar among the young and aged ............... 25
2.5 Cecal slurry bacteria maintain bioactivity following different storage at -80°C .......................................................... 27
2.6 CS-induced sepsis produces age-dependent mortality .......................... 28
2.7 Amount of circulating bacteria correlates with mortality ...................... 29
2.8 Injection of heat-inactivated cecal slurry (CS) induces endotoxemia but not mortality .......................................................... 30
3.1 Imipenem prevents the growth of cecal slurry-containing bacteria at physiological concentrations ............................................. 39
3.2 Survival rate and disease severity correlate with time of intervention after CS injection .......................................................... 42
3.3 Late intervention with antibiotics and fluid resuscitation rescues the majority of mice from otherwise lethal sepsis ............................. 44
3.4 Mild temperature regulation did not further improve survival in mice which received antibiotic and fluid resuscitation ................. 45
3.5 Further delay in therapeutic intervention with antibiotics and fluid resuscitation to 24 hours post-CS injection results in 50% survival 47
3.6 Early, but not late, therapeutic intervention blunts CS-induced cytokinemia .......................................................... 49
3.7 Late, but not early, therapeutic intervention results in prolonged reduction in body weight ..................................................... 50
3.8 Abscesses were detected in surviving mice two weeks after sepsis induction by cecal slurry with late therapeutic intervention .......... 51
3.9 Early, but not late, therapeutic intervention prevents sepsis-induced lung injury .......................................................... 52
3.10 Delayed therapeutic intervention results in heightened plasma ALT levels .......................................................... 53
3.11 Early vs. Late therapeutic intervention: A comparison of sepsis outcomes after severe infection in murine models ................. 58
4.1 Sepsis-induced muscle weakness results in loss of independence ......... 61
4.2 Delayed but aggressive therapeutic intervention rescues the majority of middle-aged animals from otherwise lethal sepsis ........................................ 65
4.3 Animals rescued from lethal sepsis using our resuscitation protocol exhibit chronic muscle weakness ............................................................... 67
4.4 Female mice develop chronic muscle weakness after sepsis .................. 68
4.5 Sepsis-induced muscle weakness is not observable using behavioral assessments of rodent strength .................................................. 69
4.6 Spontaneous wheel running activity is reduced after sepsis ................. 70
4.7 Sepsis induces long-term reductions in cage activity ......................... 71
4.8 Circadian rhythm is unchanged in sepsis survivors but sleep bout length is increased ............................................................. 72
4.9 Lean mass is reduced during sepsis but recovers by day 14 after cecal slurry (CS) injection ................................................................. 74
4.10 Skeletal muscle wet weight is reduced during sepsis but recovers to control values by day 14 after cecal slurry (CS) injection ................. 76
4.11 Skeletal muscle wet weight is reduced during sepsis but recovers to control values by day 14 after cecal slurry (CS) injection ................. 77
4.12 Abnormal pathology is evident in skeletal muscles of murine sepsis survivors ................................................................. 79

5.1 Sepsis surviving mice have increased mitochondrial volume density and disrupted mitochondrial integrity compared to non-sepsis controls ........................................................................... 87
5.2 Mitochondrial DNA is not damaged in murine sepsis survivors' skeletal muscle ................................................................................. 88
5.3 Mitochondrial respiration is impaired in sepsis-surviving mice ........... 90
5.4 Mitochondrial enzyme activities are progressively reduced over time in sepsis survivors ......................................................................... 92
5.5 Energy availability is reduced in the skeletal muscle of sepsis survivors ......................................................................................... 93
5.6 Elevated oxidative damage is evident in skeletal muscle of sepsis survivors long after bacterial clearance ........................................... 95
6.1 Skeletal muscle tissue of MnSOD-TG animals has nearly 3-fold higher expression of MnSOD ........................................................................... 103
6.2 MnSOD deficient mice have reduced MnSOD expression in skeletal muscles ......................................................................................... 107
6.3 Variable states of autophagy are present in skeletal muscle of sepsis survivors ................................................................................... 109
7.1 Protocol for preparation of cecal slurry (CS) for cryopreservation ......... 113
7.2 A mouse performing the inverted hanging test as an assessment of muscle endurance and coordination ................................................. 123
7.3 Primer design for PCR-based assay of mitochondrial DNA damage .... 131
CHAPTER 1
Introduction and Hypothesis

1.1 Sepsis

1.1.1 Pathogenesis of sepsis

Sepsis is a life-threatening condition that develops when the immune system fails to resolve a local infection, and the infection spreads. The most common primary source of infection is pneumonia (i.e. respiratory infections; 29% of cases), with others being urinary tract infections (i.e. genitourinary infections; 20.5%) and gastrointestinal infections (15.1%; Martin et al 2006). Additionally, complications from surgical procedures also contribute to sepsis cases (Elixhauser et al 2011), as do infections which develop after trauma or burn injury. Infections with antibiotic resistant bacteria are becoming increasingly more common causes of sepsis, where Methicillin-resistant Staphylococcus aureus (MRSA) is the second most commonly identified organism in sepsis cases (Elixhauser et al 2011). Many cases are polymicrobial in nature, as opposed to being caused by a single organism (Angus et al 2001; Esper et al 2006; Starr & Saito 2013). Sepsis can also result from non-bacterial sources of infection, including viral and fungal infections insults (Bone et al 1992; Martin et al 2006). In approximately half of sepsis cases, however, the specific pathogen is never identified (Elixhauser et al 2011).

Sepsis is often termed an “infection of the bloodstream,” which is the definition of bacteremia. However, sepsis is further characterized by the progressive dysregulated host immune response to the infectious stimulus which results in systemic inflammatory response syndrome (SIRS). Profound elevation of proinflammatory cytokines cause platelet activation and damage to endothelial cells, resulting in coagulation and hypotension. If not resolved, the condition progresses to severe and often times irreversible tissue damage due to hypoxia and oxidative stress, which contribute to the development of multi-organ failure (MOF) and death (Figure 1.1).
Figure 1.1. Progression of sepsis pathogenesis. The sepsis condition results when the immune system fails to contain a local infection, which spreads to the bloodstream (i.e. bacteremia) and triggers a profound inflammatory response, characterized by strikingly elevated levels of proinflammatory cytokines. Left unresolved, coagulation and edema contribute to the development of hypotension, often causing irreversible tissue damage, including in the skeletal muscle. Ultimately these factors can contribute to the development of multi-organ failure and subsequent death. *Adapted from Buras et al 2006.*

As such, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer *et al* 2016). Clinically, organ dysfunction is identified as an increase in the Sequential Organ Failure Assessment (SOFA) score of ≥ 2 points (Seymour *et al* 2016; Singer *et al* 2016). Septic shock is a subset of sepsis cases in which patients have severe circulatory, cellular, and metabolic abnormalities which are associated with increased sepsis-associated mortality (Shankar-Hari *et al* 2016) which occurs in approximately 20% of sepsis patients (Shankar-Hari *et al* 2017; Angus *et al* 2001). Patients with septic shock are identified by having hypotension which requires vasopressor therapy to maintain mean blood pressure of 65 mmHg or
greater, and having serum lactate level of > 2 mmol/L after adequate fluid resuscitation (Shankar-Hari et al 2016).

1.1.2 Sepsis epidemiology and survival outcomes

Every year, nearly 1.7 million patients are diagnosed with sepsis in the US when taking into account both primary and secondary diagnoses (836,000 and 829,000 cases, respectively; Elixhauser et al 2011). The majority of sepsis cases and deaths are among the elderly population, as both incidence and mortality increase with age (Martin et al 2003). The incidence rate is increasing by approximately 9 to 13% annually (Martin et al 2003; Gaieski et al 2013; Angus et al 2001) with an average of 4,600 new patients being treated for sepsis every day (Elixhauser et al 2011). This increase in incidence exceeds the rate of population expansion, and is attributed to a variety of factors including increased ability to diagnose the condition, rising trend of invasive surgical procedures, an expanding elderly population (explained further below in Section 1.4), as well as increased population with immune disorders and heightened risk of antibiotic resistant bacteria (Martin et al 2006).

Although the sepsis incidence rate continues to climb, improved case definitions (i.e. diagnosis criteria) and advances in critical care medicine have led to a decline of the mortality rate in recent decades (Angus et al 2001). The sepsis mortality rate remains high compared to stays in the ICU for other conditions (approximately 8-times higher; Elixhauser et al 2011). Thus, a significant amount of work remains to further understand the pathophysiology of sepsis to develop pharmacological interventions and further decrease the short-term mortality rate (Angus et al 2001). It has also become a priority to understand the challenges of sepsis survivors after hospital discharge. The combination of the increased incidence and decreased mortality resulted in over 1.5 million sepsis survivors discharged from US hospitals in 2012; which is more than two-fold higher compared to the previous decade (Figure 1.2 B; Elixhauser et al 2011; Stoller et al 2016).
Figure 1.2. The number of sepsis survivors in the US has doubled within the past decade. The sepsis incidence rate has continued to rise, whereas the mortality rate has steadily decreased over recent years (A; reproduced from Stoller et al. 2016). Such trends have resulted in a doubling of the number of sepsis survivors being discharged from US hospitals, illustrated by number of discharges in 2012 compared to 2000 (B). Data adapted from Elixhauser et al. 2011 and Stoller et al. 2016.

1.2 Chronic muscle weakness after sepsis

1.2.1 Muscle weakness in patients with sepsis during their stay in the ICU

Physicians first reported muscle weakness in critically ill patients with sepsis and multi-organ failure (MOF) three decades ago (Bolton et al 1984). Since then, this phenomenon has been demonstrated using a variety of methods. Lanone et al. showed significant reductions in sub-maximal (30-Hz stimulation) force generation capacity in rectus abdominus muscle biopsies obtained from septic patients compared to biopsies taken from non-sepsis controls who were undergoing elective laparotomy or cardiac surgery (Lanone et al 2000). Eikermann et al. conducted standard electrophysiological tests in the adductor pollicis muscle (located in the hand) by ulnar nerve stimulation in sepsis patients.
with multi-organ failure (MOF) and age-matched healthy controls whose arm and thumb were immobilized for 14 days using a lower arm cast. This group found that sepsis patients had a 30% reduction in force generation capacity compared to immobilized controls, demonstrating that disuse alone cannot explain sepsis-induced muscle weakness (Eikermann et al 2006). Baldwin and Bersten (2014) evaluated strength in patients with sepsis who had been mechanically ventilated for at least 5 days during their hospital stay. They analyzed peripheral muscle strength by isometric handgrip test and elbow flexion, both of which were significantly reduced in sepsis patients with a similar trend in knee extension force compared to age and sex matched healthy controls. Although each of these studies has limitations, they clearly indicate that sepsis induces severe muscle weakness.

Intensive care unit-acquired weakness (ICUAW) and muscle wasting are complications which arise due to immobilization during bedrest, sedation, and disuse, as well as specific pharmacological interventions and fasting (Schefold & Bierbrauer 2010). However, sepsis is further complicated due to its pathophysiology and pharmaceutical interventions, leading to the development of critical illness myopathy (CIM; Eikermann et al 2006). More specifically, elevation of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) contribute to impaired insulin resistance which has been linked to decreased mammalian target of rapamycin (mTOR) signaling and therefore decreased skeletal muscle protein synthesis (Spranger et al 2003; Frost et al 2005; Frost et al 2008; Lang et al 2007; Schefold & Bierbrauer 2010). Sepsis-associated cytokinemia not only decreases protein synthesis (Lang et al 2007; Vary & Kimball 1992), it also stimulates protein degradation through multiple pathways, including lysosomal degradation (Ebisui et al 1995), ubiquitin proteasome (Klaude et al 2007), and apoptotic pathways (Comstock et al 1998). To further complicate the matter, many patients require mechanical ventilation, further causing disuse-mediated atrophy and weakness (Callahan & Supinski 2009). These changes in muscle protein synthesis and breakdown result in muscle wasting beyond that which is seen in the ICU for other conditions,
however nutritional support aimed at stimulating protein synthesis does not effectively restore balance (Lang et al 2007; Schefold & Bierbrauer 2010; Ash & Griffin 1989; Minnaard et al. 2005). Further, animals which are pair-fed to animals with sepsis do not have muscle weight loss (Voisin et al 1996), demonstrating that therapeutic interventions beyond nutritional supplementation and more specific to sepsis pathophysiology are necessary to prevent and/or recover muscle weakness in patients with sepsis.

Beyond sepsis-associated imbalances in protein degradation and synthesis, sepsis-mediated inflammation, as well as tissue hypoxia caused by coagulation and vascular endothelium damage, trigger the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which cause severe oxidative damage to many cell types, including the motor unit (Lanone et al 2000; Singer 2014; Fredriksson et al 2008). Oxidative damage to the peripheral nerve and/or neuromuscular junction results in polyneuropathy, also referred to as critical illness polyneuropathy (CIP), which results in desensitization of sensory nerve amplitudes and thus decreased force (Garnacho-Montero et al 2001). Although many cases of polyneuropathy have been reported, it only occurs in a small fraction of patients (Koch et al 2011).

Being that skeletal muscle is highly oxidative (the largest consumer of oxygen in the body), generation of free radicals is more pronounced compared to other tissues (Doria et al 2012; Fulle et al 2014). Therefore, it is not surprising that sepsis-induced oxidative stress also causes damage to numerous sites on the myofibrillar level that may result in reduced force generation capacity in sepsis patients. The proinflammatory cytokine TNFα has been shown to trigger free radical production that causes damage to sarcomeric (i.e. contractile) proteins, which hinders muscle contraction and force production (Callahan et al. 2001; Hardin et al 2008; Callahan & Supinski 2009). This is of particular concern since skeletal muscle proteins scavenge as much as 50-75% of ROS during acute oxidative stress, inducing structural changes to actin and isoforms of myosin heavy chain (Dalle-Donne et al 2003), likely having large impacts on muscle contraction.
Sepsis-induced oxidative stress also damages skeletal muscle mitochondria (Mela 1979; Singer 1999; Boveris et al 2002). Such damage results in impaired mitochondrial function (i.e. reduced oxidative phosphorylation) as Fredriksson et al. (2006) showed in patients with sepsis and MOF who had 40% lower levels of adenosine triphosphate (ATP) in leg muscle (vastus lateralis). Complex I-driven electron transport chain activity (i.e. nicotinamide adenine dinucleotide (NAD) dehydrogenase activity) is more impaired by sepsis than is complex-II driven activity (i.e. succinate dehydrogenase (SDH) activity; Poderoso 1978; Boveris 2002). These reports are supported by ex vivo experiments in which isolated mitochondria cultured with nitric oxide synthase (NOS) decreased respiration rates, whereas culturing with NOS-inhibitors resulted in increased respiratory rates (Giulivi et al 1998; Boveris et al 2002), demonstrating the effect of oxidative stress on mitochondrial function.

Fredriksson later reported in 2008 that reduction in mitochondrial respiration is not associated with differences in mitochondrial enzyme subunits. Instead, they found that mitochondrial biogenesis was upregulated in sepsis patients, but there was a loss of its coordination, leading to immature mitochondrial networks and increased protease activity. Not surprisingly, the mitochondria were also characterized by low levels of reduced glutathione (GSH), indicating that endogenous mitochondrial anti-oxidant capabilities are exhausted during sepsis (Brealey et al 2002). With reduced anti-oxidant capacity, mitochondria are further exposed to oxidative damage, especially complex I, which is especially susceptible to damage by RNS (Bolanos et al 1997). Further, since mitochondria produce ROS as a byproduct of oxidative phosphorylation, damaged mitochondria generate an abundance of free radicals, causing additional damage to mitochondria and nearby proteins. Since muscle contraction is an ATP-dependent process, reduced energy availability results in reduced muscle force generation capacity.

Also, the sarcoplasmic reticulum is subject to oxidative damage because the skeletal muscle ryanodine receptor is highly reactive to nitric oxide and hydrogen peroxide (Hamilton et al 2000). Release of calcium from the
sarcoplasmic reticulum upon efferent signaling is essential for muscle function whereby it binds troponin on the thin filament, exposing the myosin binding site and allows the cross-bridge cycle to occur for muscle contraction. Thus, oxidative damage to the sarcoplasmic reticulum may alter calcium dynamics and result in reduced muscle contraction in sepsis patients (Cohen et al. 2006; Callahan & Supinski 2009).

In addition to the complex pathophysiology of sepsis which contribute to development of muscle weakness, pharmacological interventions may also play a role in this phenomenon (Schefold & Bierbrauer 2010). Although controversial, treatment with glucocorticoids has been implicated as a predictor of muscle weakness (de Jonghe et al. 2002), which other groups suggest is a dose-dependent issue (Weber-Carstens et al. 2010). On the other hand, use of neuromuscular blockers has also been associated with increased risk for development of muscle weakness (Segredo et al. 1992), but its involvement is also not well understood (de Jonghe et al. 2009; Schefold & Bierbrauer 2010).

Thus, sepsis pathogenesis and pharmacological treatments likely further propagate muscle weakness beyond what is mediated through disuse alone. Sepsis-induced muscle weakness is a complex situation in which numerous factors may contribute to the development of decreased muscle contraction, including suppressed protein synthesis, increased protein degradation, oxidative damage to multiple organelles and contractile proteins, and treatment with corticosteroids and neuromuscular blockers which seem to further increase risk of the development of muscle dysfunction.

1.2.2 Persistent muscle weakness and chronic disability in sepsis survivors

Not only do sepsis patients have significant muscle weakness, it is now largely acknowledged that the majority of patients who survive septic shock have prolonged muscle weakness that persists for years (Callahan & Supinski 2009). Koch et al. (2011) showed that upon hospital discharge, the majority of patients did not recover muscle strength as evaluated using the Medical Research Council (MRC) scale. Strikingly, sepsis-induced muscle weakness is so
substantial that 23% of sepsis survivors who were functionally independent prior to becoming septic developed functional dependency and were discharged to nursing care facilities, and an additional 23% were discharged home with home care (Odden et al 2013). Patients who had limitations in activities of daily living (ADLs) prior to hospitalization for sepsis were at an even higher risk for being discharged to nursing care facilities (56%, p =0.009 vs. patients with no previous functional dysfunction; Odden et al 2013). Heyland et al. (2000) found that sepsis survivors had significantly reduced health-related quality of life (HQRL) scores, especially in terms of physical functioning, general health, and vitality, two-weeks after hospital discharge. Even further, Iwashyna et al. (2010) conducted a prospective study of over 500 sepsis patients and found that patients continued to develop functional limitations during the 5-year-long study following hospital discharge, a trend which was not observed in non-sepsis cases. ADLs include ability to walk, dress, bathe, prepare meals, use the restroom or go to bed without assistance, as well as use the telephone, take medications, and manage money; thus development of limitations in any singular area would be life-altering, and development of multiple limitations is especially devastating, as is the case for septic shock survivors (Iwashyna et al 2010).

Battle et al. (2014) recently sought to evaluate the influence of sepsis severity on long-term (6 months to two years) physical/functional health. They conducted a prospective study which controlled for age, length of stay in the ICU, and comorbidities, and compared physical and cognitive outcomes in three groups: patients with sterile SIRS, patients with uncomplicated sepsis (i.e. without evidence of organ dysfunction), and patients with septic shock. Evaluation of physical function, bodily pain, general health, and vitality (assessed using a standard health survey (short form-12)) showed that the septic shock survivors had significantly lower scores compared to both the sterile SIRS group and patients with uncomplicated sepsis, as demonstrated in Figure 1.3. These data clearly show that patients with more severe cases of sepsis are at higher risk for developing functional limitations during hospitalization as well as in the years following recovery from sepsis pathogenesis itself.
Figure 1.3. Increased sepsis severity is associated with more profound reductions in health-related quality of life. Patients with sterile systemic inflammatory response syndrome (SIRS), uncomplicated sepsis, and septic shock were recruited to take a survey within 6 months to 2 years following hospital discharge to evaluate physical and mental health. Results from the physical components of that survey are shown, with data expressed as means ± SD. † signifies p<0.05 SIRS vs. uncomplicated sepsis, ♦ signifies p<0.05 SIRS vs. septic shock, and # signifies p<0.05 uncomplicated sepsis vs. septic shock. This work is adapted from Battle et al. 2014 used under international license CC BY 4.0.
1.3 Lack of an appropriate animal model as limitation of previous studies

The scientific community acknowledges that sepsis-induced muscle weakness is a problem. However, the field lacks an animal model of sepsis that induces organ damage but also high survival, which has been a critical barrier to identifying the mechanisms responsible for long-term muscle dysfunction. The first major problem with current animal models is that they are either severe models causing early death of most animals, or are mild models which only cause weakness during sepsis, neither of which are appropriate to understand mechanisms of long-term sepsis-induced muscle dysfunction (Figure 1.4). With existing models, the majority of research in the field has focused on mechanisms of atrophy (i.e. muscle wasting) during the acute phase, and the small number of studies which are conducted in late phases do not include functional analyses (Table 1.1), thus no associations can be made about actual muscle weakness.

![Experimental sepsis diagram]

**Figure 1.4.** The lack of an appropriate animal model of sepsis has been a critical barrier to evaluating mechanisms of long-term muscle weakness in sepsis survivors. Induction of mild sepsis results in high survival but not organ injury (left arm). However, induction of severe sepsis without therapeutic intervention results in early death, and therefore long-term studies cannot be performed (middle arm). We hypothesized that if severe sepsis was induced, and therapeutic intervention was delayed but conducted similarly to ICU-care, the majority of animals would survive and long-term studies could be performed (right arm).
### Table 1.1 Muscle studies using current animal models of sepsis

<table>
<thead>
<tr>
<th>Model</th>
<th>Dose/severity</th>
<th>Species</th>
<th>Time-point(s)</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endotoxemia (lipopolysaccharide)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>10 mg/kg i.p.</td>
<td>Rat</td>
<td>2, 6, &amp; 12 hours</td>
<td>↑ myofibrillar proteolysis (EDL)</td>
<td>Chai et al. 2003</td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ mRNA for ubiquitin (EDL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>12 mg/kg i.p.</td>
<td>Rat</td>
<td>48 &amp; 96 hours</td>
<td>↑ mass &amp; protein content (diaphragm; 96 h)</td>
<td>Supinski et al. 2009</td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ proteolysis (48 &amp; 96 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>3 mg/kg i.p.</td>
<td>Rat</td>
<td>6, 24, 48, 72 hours</td>
<td>↑ muscle mass &amp; protein content (EDL, TA) (at 24, 48, and 72 h)</td>
<td>Macallan et al. 1996</td>
</tr>
<tr>
<td>E. coli</td>
<td>3 mg/kg s.c.</td>
<td>Rat</td>
<td>6 to 72 hours</td>
<td>↑ protein synthesis (maximum at 30 h; GM)</td>
<td>Jepson et al. 1986</td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ proteolysis (48 &amp; 96 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>0.08 mg/kg i.v.</td>
<td>Rat</td>
<td>18 hours</td>
<td>↑ proteolysis (soleus, EDL)</td>
<td>Ash &amp; Griffin 1989</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>2 mg/kg i.p.</td>
<td>Rat</td>
<td>3.5 hours</td>
<td>↑ protein synthesis (GM)</td>
<td>Holecek et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ whole-body proteolysis</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>1 mg/kg i.p.</td>
<td>Mouse</td>
<td>12 &amp; 18 hours</td>
<td>↓ mass &amp; protein content (GM; 18 h)</td>
<td>Jin &amp; Li 2007</td>
</tr>
<tr>
<td>&amp;</td>
<td>↓ mRNA for ubiquitin (GM; 12 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>25 µg/animal</td>
<td>Mouse</td>
<td>4 hours</td>
<td>↓ protein synthesis (GM)</td>
<td>Lang et al. 2010</td>
</tr>
<tr>
<td>E. coli</td>
<td>10 mg/kg i.p.</td>
<td>Mouse</td>
<td>24 hours</td>
<td>↑ atrogin-1 &amp; MuRF1 expression (ubiquitin)</td>
<td>Kim et al. 2012</td>
</tr>
<tr>
<td>Zymosan</td>
<td>300 mg/kg i.p.</td>
<td>Rat</td>
<td>2 hours, 2, 6, &amp; 11 days</td>
<td>no effect on specific force</td>
<td>Minnaard et al. 2004</td>
</tr>
<tr>
<td>Not provided</td>
<td></td>
<td>Hamsters</td>
<td>72 hours</td>
<td>↓ dose-dependent force production (diaphragm, FHL)</td>
<td>Supinski et al. 1996</td>
</tr>
<tr>
<td>Live bacteria administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>i.v.</td>
<td>Rat</td>
<td>2, 6, 10 days</td>
<td>↑ muscle mass (soleus, EDL, TA) Activation of ubiquitination and lysosomal proteolysis</td>
<td>Voisin et al. 1996</td>
</tr>
<tr>
<td>S. aureus</td>
<td>i.m.</td>
<td>Mouse</td>
<td>24 hours</td>
<td>↑ proteolysis (GM)</td>
<td>Khal &amp; Tisdale (2008)</td>
</tr>
<tr>
<td>E. coli &amp; B. fragilis</td>
<td>Fecal-agar pellet</td>
<td>Rat</td>
<td>5 days</td>
<td>↓ protein synthesis (GM, not soleus)</td>
<td>Vary &amp; Kimball 1992</td>
</tr>
<tr>
<td>Cecal slurry administration (polymicrobial)</td>
<td>Diluted CS</td>
<td>Mouse</td>
<td>≤ 72 hours</td>
<td>↓ whole-body metabolism</td>
<td>Zolfaghari et al. 2015</td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ force production (diaphragm, 24 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp;</td>
<td>↓ MT membrane potential (diaphragm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecal ligation and puncture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>16 hours</td>
<td></td>
<td>↑ proteolysis (EDL)</td>
<td>Fisher et al. 2000; Fareed et al. 2006</td>
<td></td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ proteolysis (EDL)</td>
<td></td>
<td>No effect on protein synthesis (soleus, EDL)</td>
<td>Safranek et al. 2006</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>18 hours</td>
<td></td>
<td>↑ proteolysis (soleus, EDL)</td>
<td>Kadlicikova et al. 2004</td>
<td></td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ proteolysis (soleus, EDL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>5 days</td>
<td></td>
<td>↑ mass &amp; protein content (GM)</td>
<td>Nystrom et al. 2009</td>
<td></td>
</tr>
<tr>
<td>&amp;</td>
<td>↑ atrogin-1 &amp; MuRF1 expression (ubiquitin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>7 days</td>
<td></td>
<td>mass (GM)</td>
<td>Lang et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>≤ 48 hours</td>
<td></td>
<td>↓ inspiratory pressure (6 hours)</td>
<td>Peruchi et al. 2011</td>
<td></td>
</tr>
</tbody>
</table>

i.p., intraperitoneal; EDL, extensor digitorum longus; TA, tibialis anterior; GM, gastrocnemius; FHL, flexor hallucis longus. *Adapted from Holecek 2012, with additions of studies in blue rows.*
Some protocols include antibiotic regimens, but are often initiated very soon after induction of the infection, and thus block the progression of sepsis pathogenesis and stray very far from the clinical situation. It is important to note that in the treatment of clinical sepsis, antibiotics are administered as early as possible after recognition of sepsis or septic shock as per the guidelines of the Surviving Sepsis Campaign (Dellinger et al 2008). Retrospective analysis of 165 ICUs in Europe, the United States, and South America showed that 26.3% of patients were treated within the first hour, and the majority (68.6%) of patients was treated within three hours after recognition of sepsis condition. This was based on the wide evidence that delay in treatment with antibiotics is linearly correlated with increased in-hospital mortality (Kumar et al 2006; Gaieski et al 2010; Ferrer et al 2014). However, this early intervention is after recognition of sepsis, not after initiation of infection; therefore intervention in animal models should be based on timing after development of sepsis, not after infectious insult. Other protocols include antibiotic therapy initiated at a late time-point, but do not include other therapeutic strategies as used clinically, and see minimal changes in survival (Turnbull et al 2003). Therefore these models mimic more of a local infection or SIRS phenotype rather than septic shock. When long-term studies are performed using these models, molecular analyses are performed without functional analysis of muscle strength, therefore associations cannot be made.

In addition to lack of therapeutic intervention in other models, many studies use the cecal ligation and puncture (CLP) surgical model of sepsis, which we believe is not appropriate for long-term studies. Instead, we used the cecal slurry (CS) injection model to induce polymicrobial abdominal sepsis for multiple reasons: (i) mice that survive CLP-induced sepsis have a ligated cecum that may affect dietary habits and GI function, in addition to unresolved necrosis which may introduce variability in long-term physiological processes; (ii) cecum shape is often highly variable among older mice (Starr, Steele et al 2014), which causes variability in ligation site; (iii) the severity of sepsis can be more precisely and accurately controlled using this CS-model (by controlling the injection volumes) than CLP-model can control (by puncture size or number); (iv) the CS-injection
model does not require anesthesia and surgery, and thus is highly reproducible and a larger number of mice can be investigated in a shorter time.

Therefore, currently existing models do not mimic the clinical situation, especially when experimental questions concern long-term outcomes of septic shock survivors. With the lack of an ICU-like resuscitation protocol, and with the majority of current studies using the CLP model which introduces multiple concerns as noted above, progression in the field has been blunted despite recognition of muscle weakness as a clinical problem in sepsis survivors.

1.4 Significance of using middle-aged animals

In addition to the limitations of current sepsis animal models discussed above, another shortcoming of the majority of studies is the lack of consideration for age of animals used. Sepsis is a disease of the aged, where advanced age is an established risk factor for both incidence and mortality (Figure 1.5; Martin et al 2006; Elixhauser et al 2011; Angus et al 2001; Dombrovskiy et al 2007). This age-associated susceptibility may be in part due to decreased immune function in the aged, including failed antigen processing and altered inflammatory responses including failed antigen processing by leukocytes (Pawelec et al 1998) and altered inflammatory cytokine expression (Miller et al 1996; Tateda et al 2006; Martin et al 2006). Further, age is associated with coagulation abnormalities (Starr & Saito 2014; Cohen et al 2003; Mari et al 1995). The incidence rate for sepsis in the middle-aged (ages 45-64) is nearly four times higher than in younger adults (ages 18-44) (Elixhauser et al 2011), and the incidence rate is increasing disproportionately with age compared to young adults (20.4% faster) (Martin et al 2006).
Figure 1.5. Risk of sepsis incidence and mortality increase with advancing age. Incidence rate (left abscissa) and case-fatality rates (right abscissa) for sepsis are adjusted and stratified by age deciles over a 24-year (from 1979-2002; >10 million adult sepsis patients) study period. Data is represented as means ± SEM. Martin et al. 2006.

1.5 Study rationale

1.5.1 Critical barrier to progress in the field

The scientific community acknowledges that sepsis-induced muscle weakness is a serious problem, especially as the population of sepsis survivors grows by > 1 million every year. Muscle weakness during sepsis in humans and animal models, with special emphasis on atrophic mechanisms, has been studied extensively. However, the scientific community lacks a severe animal model of sepsis that can be used to study chronic pathophysiologies; mild sepsis models do not exhibit chronic muscle weakness, whereas severe sepsis models cause early death of animals and thus prevent long-term studies. The lack of an appropriate model has been a critical barrier to the identification of factors that are involved in muscle weakness after sepsis. This has greatly prevented progress in elucidating therapeutic targets to prevent and/or recover muscle strength in sepsis survivors and allow patients to return to normal productive lives.
1.5.2 Central hypothesis

We hypothesized that if lethal sepsis was induced, and ICU-like therapeutic interventions were performed after development of bacteremia, then sepsis survivors with long-term muscle weakness would be achieved and then utilized to elucidate the underlying responsible mechanism(s).

1.5.3 Specific aims

In pursuit of this dissertation project, two specific aims were set:

**SPECIFIC AIM 1:** To develop a clinically relevant non-lethal severe model of sepsis that results in chronic muscle weakness. Under this aim, the protocol for preparation of cecal slurry (CS) was refined so that large batches could be frozen for long-term storage without reduced bacterial bioactivity (Chapter 2) which ensured efficiency and reproducibility in the later parts of this project. The CS injection model of polymicrobial sepsis was then used in young animals for development of a novel delayed-intervention ICU-like aggressive resuscitation protocol which rescues the majority of animals from otherwise completely lethal sepsis (Chapter 3). Third, this CS injection/ICU-like resuscitation protocol was adapted for middle-aged animals, and muscle strength was assessed in sepsis survivors two weeks later using ex vivo analysis (Chapter 4).

**SPECIFIC AIM 2:** To characterize long-term muscle weakness in sepsis survivors on the sub-cellular level. Under this aim, we first identified that myopathy, rather than atrophy or polyneuropathy, is responsible for post-sepsis muscle weakness in murine sepsis survivors (Chapter 4). Next, skeletal muscle mitochondrial integrity was assessed by transmission electron microscopy, function was evaluated by respiration analysis and histochemical assays, and markers of oxidative damage were measured (Chapter 5). The results of this aim indicate that sepsis-mediated changes in muscle quality rather than quantity are responsible for chronic muscle weakness in sepsis survivors.
Portions of this chapter were adapted and reprinted by permission from:


CHAPTER 2

Refinement of the Cecal Slurry Model of Experimental Sepsis

2.1 Abstract

Sepsis is widely studied using laboratory animal models, most often using the cecal-ligation and puncture (CLP) surgical model of abdominal sepsis. Although it is considered the gold standard model for sepsis research, it may not be preferable for experiments comparing animals of different size or under different dietary regiments. By comparing cecum size, shape, and cecal content characteristics in mice under different experimental conditions (aging, diabetes, pancreatitis), we show that cecum variability could be problematic for some CLP experiments. Additionally, the CLP model of sepsis is not appropriate for long-term studies on sepsis survivors due to ongoing necrosis of the ligated cecum which is not controlled for as an experimental variable. The cecal slurry (CS) injection model, in which the cecal contents of a laboratory animal are injected intraperitoneally to other animals, is an alternative method for inducing polymicrobial sepsis; however under conventional protocols the CS must be freshly prepared which is a major disadvantage with respect to reproducibility and convenience. The objective of this study was to develop an improved CS preparation protocol that allows for long-term storage of CS with reproducible results upon injection. Using our new CS preparation protocol we found that bacterial viability is maintained for at least 6 months when the CS is prepared in 15% glycerol-PBS and stored at -80°C. To test sepsis-inducing efficacy of stored CS stocks, various amounts of CS were injected to young (4-6 months old), middle-aged (12-14 months old), and aged (24-26 months old) male C57BL/6 mice. Dose- and age-dependent mortality was observed with high reproducibility. Circulating bacteria levels strongly correlated with mortality suggesting an infection-mediated death. Further, injection with heat-inactivated CS resulted in acute hypothermia without mortality, indicating that CS-mediated death is not due to endotoxic shock. This new CS preparation protocol results in CS stocks which
are durable for freezing preservation without loss of bacterial viability, allowing experiments to be performed more conveniently and with higher reproducibility than before.

2.2 Introduction

Among various types of animal models for sepsis research, cecal ligation and puncture (CLP) is one of the most frequently used procedures to induce experimental sepsis in laboratory animals such as mice and rats (Dejager et al 2011, Wichterman et al 1980, Hubbard et al 2005). Under the CLP model, polymicrobial peritonitis is induced in anesthetized animals by surgical ligation of the cecum followed by needle puncture to secrete cecal contents into the abdominal cavity. The CLP model has been preferred by many investigators who study sepsis using laboratory animals because it is a relatively simple surgical procedure and closely mimics the clinical course of intra-abdominal sepsis (Dejager et al 2011).

However, the severity of CLP-induced sepsis is highly dependent on the degree of infection which can be influenced by volume, rapidity, and duration of cecal content released into the abdomen, and by the bacterial flora present in the cecum. Therefore, despite its popularity, CLP may not be preferable for certain experiments including those investigating animals with different cecum size, shape, or bacterial flora. This concern may apply for studies that compare severity of sepsis among animals with different body size (e.g. neonatal mice or mutant dwarf mice), under different diet regimens (e.g. liquid diet, high fat diet, or diet restriction), different gastrointestinal conditions (e.g. neonatal or aged animals or animals with gastrointestinal pathology), or increased sensitivity to surgery (e.g. aged animals or animals with deficient wound-healing capability).

Additionally, the CLP model is not appropriate for long-term studies on sepsis survivors. Animals that survive CLP-induced sepsis have a ligated cecum that may affect dietary behaviors and GI function. Further, the ligated cecum undergoes necrosis which likely potentiates an inflammatory response. Although
control animals undergo a sham operation, the cecal ligation is rarely performed. Therefore, CLP sepsis survivors often have unresolved necrosis and ongoing inflammation that is not controlled for among groups, making it impossible to determine if any difference in phenotype across groups is due to sepsis itself or due to the ongoing influence of the ligated cecum.

In cases when CLP is not preferable, intraperitoneal injection of cecal slurry (CS) is an alternative method. In the CS model, contents from the cecum of donor animal(s) are suspended in liquid form and injected into the abdominal cavity of other animals to induce polymicrobial sepsis (Gentile et al 2014, Wynn et al 2007, Shrum et al 2014, Gentile et al 2014, Lang et al 1983). The CS model of sepsis has been preferred by a limited number of investigators, particularly those who study sepsis in neonatal mice. However, one prominent disadvantage of the CS model is that the currently accepted protocol for CS preparation with 5% dextrose in water (D5W) does not allow for long-term storage, and thus the CS has to be freshly prepared each time an experiment is performed (Sam et al 1997). This can lead to significant variability from experiment to experiment. Thus, the first set of experiments under this project was aimed at circumventing this problem. We developed a new CS preparation procedure that allows for the long-term storage of CS stocks without loss of bacterial viability. In addition, we validated survival rates of mice at different ages using stored CS prepared with this new protocol.

2.3 Experimental approach

To evaluate potential differences in cecum characteristics across experimental models, cecums from young (4-6 month-old) healthy mice were compared to those from animals with chronic pancreatitis, type I diabetes, or aged (24-26 month-old) animals. To induce chronic pancreatitis in mice, recurrent acute pancreatitis was induced in young mice (6-months old) by a procedure modified from our previous protocol for acute pancreatitis (Okamura et al. 2012). Each mouse received intraperitoneal (i.p.) injection with either physiological saline (control) or caerulein at a dose of 50 μg/kg body weight, 6-
times hourly, 3 days per week for 9 weeks. To induce diabetes, fasted mice received streptozotocin at a dose of 45 mg/kg body weight, i.p. once daily for 5 consecutive days. Control mice received no injection.

For cecum analysis, at sacrifice, ceca were dissected from mice using sterile instruments. Each cecum was placed in a plastic container, weighed, and photographed. The contents of each cecum were collected using sterile forceps and spatula, weighed (wet weight), and dried in an oven at 60°C for 48h (dry weight). A wet/dry ratio was calculated for the contents of each cecum.

To assess the aerobic cecal bacteria, immediately after collection, cecal contents from young and aged mice were suspended in sterile water at a concentration of 5 mg/mL. The resulting suspension was mixed well and 50 μL was spread onto multiple agar plates containing 3.7% w/v brain-heart infusion and 0.15% w/v agar with aztreonam for Gram-positive bacteria selection (Wood et al 1993), penicillin G for Gram-negative selection, or without antibiotics for total aerobic bacteria quantification.

2.4 Results

2.4.1 Cecum size, shape, and cecal content consistency varies widely among different experimental conditions

To show that under some experimental conditions the CLP model of sepsis is not ideal, we compared cecum size, shape, and cecal content consistency in various animal models including diabetes, chronic pancreatitis, and aging. Type 1 diabetes was induced by multiple low dose streptozotocin (STZ) injection model (confirmed by hyperglycemia), and ceca were dissected from mice at sacrifice. Cecal content wet weight were not different between the two groups (Figure 2.1 A), but cecal content wet/dry ratio (Figure 2.1 B) showed a significant difference indicating that cecal matter within the cecum of diabetic mice has a different consistency than normal mice. This could complicate CLP-induced sepsis because the watery cecal content of diabetic mice may leak into the abdomen from the puncture site much more rapidly than that of control mice.
Figure 2.1. Abnormal consistency of cecal contents in diabetic mice. Ceca and cecal contents were collected from non-treated control and streptozotocin (STZ)-induced diabetic mice. Wet weight of cecal contents (A) and wet/dry weight ratio of cecal contents (B) were measured (n=5 per group). Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.

Chronic pancreatitis was induced by repeated bouts of acute pancreatitis using caerulein injection and ceca were dissected from mice at sacrifice. Macroscopically, the ceca of mice with chronic pancreatitis appeared enlarged and fuller than that of control mice (Figure 2.2 A). Cecal content wet weight was significantly different (p<0.001) between the two groups (Figure 2.2 B) indicating that mice with chronic pancreatitis have more fecal matter in their cecum. The wet/dry ratio of cecal content between control mice and mice with chronic pancreatitis was not significantly different (Figure 2.2 C). Whole cecum weight was significantly different (Figure 2.2 D). These findings indicate that while stool consistency is similar, the enlarged ceca of mice with chronic pancreatitis could be problematic when determining which portion of the cecum to ligate during CLP procedure.
Figure 2.2. Mice with chronic pancreatitis have enlarged ceca. Macroscopic images (A), wet weight of cecal contents (B), wet/dry weight ratio of cecal contents (C), and whole cecum weight (D) from mice with caerulein-induced chronic pancreatitis and saline-injected control mice (n=5 per group). Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.
We further compared cecum size, shape, cecal content consistency, and bacterial flora in young (4-6 month-old) versus aged (24-26 month-old) mice. Ceca were dissected using sterile instruments from young and aged mice at sacrifice. Macroscopically, the ceca of young mice appear uniform in size and shape, while the ceca size and shape are more variable among aged mice (Figure 2.3 A). Despite different cecum shape and significantly different body weight in this set of young and aged mice (27.8 ± 2.2 and 36.5 ± 1.7 respectively, p<0.001), cecum wet weight, cecal content wet/dry ratio, and whole cecum weight did not show differences between the age groups (Figure 2.3 B-D).

Figure 2.3. Cecal shape is highly variable in aged mice. Macroscopic images (A), wet weight of cecal contents (B), wet/dry weight ratio of cecal contents (C), and whole cecum weight (D) from mice with caerulein-induced chronic pancreatitis and saline-injected control mice (n=5 per group). Adapted from Starr. Steele et al. 2014 used under international license CC BY 4.0.
A small portion (~100 mg) of the cecal content from each mouse cecum was taken under sterile conditions for aerobic bacterial analysis. Fecal material was dissolved in sterile water at a concentration of 5 mg/mL and spread onto agar plates with penicillin G to select for Gram-negative bacteria, Aztreonam to select for Gram-positive bacteria, or without any antibiotics for total aerobic bacterial quantification. No significant differences were found in aerobic bacteria colony formation for total bacteria, or when selecting for Gram-positive or Gram-negative bacteria (Figure 2.4). Collectively, these data indicate that while cecum shape in aged mice are more variable and differ from that of young mice, cecal content in terms of weight and bacterial flora are highly similar.

**Figure 2.4. Cecum bacterial flora are similar among the young and aged.** Cecal contents from young and aged mice were suspended in sterile water and spread onto agar plates without antibiotics, with aztreonam to select for Gram-positive bacteria, or with Penicillin G to select for the majority of Gram-negative bacteria. Data represent the mean ± standard deviation, n=5 for each group. *Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.*
2.4.2 Cecal slurry bacteria remain bioactive after long-term storage when prepared using a refined protocol

Our objective was to establish a cecal slurry preparation protocol that eliminates the prior necessity of preparing the slurry fresh the day of sepsis induction. Therefore, we refined the protocol so that cecal contents are suspended in a glycerol solution to allow for freezing of the slurry-containing bacteria (Figure 7.1). Four-month-old C57BL/6 mice were sacrificed by cervical dislocation and the whole cecum was dissected from each mouse. The cecal contents were collected using sterile forceps and spatula, and the cecal contents were combined, weighed, and mixed with sterile water at a ratio of 0.5 mL of water to 100-mg of cecal content. This cecal slurry was sequentially filtered through sterile meshes (860, 190, and 70 mm. The filtered slurry was then mixed with an equal volume of 30% glycerol in phosphate buffered saline (PBS), resulting in a final CS stock solution in 15% glycerol. While continuously stirring on a plate with a magnetic stir bar, the CS stock was dispensed into cryovials.

To determine whether bacterial viability could be maintained in stored CS samples, CS stocks were prepared in 15% glycerol buffer and stored in aliquots at 4, -20, and -80°C. One aliquot was immediately diluted and spread onto agar plates to determine colony forming ability. One and six weeks after preparation, an aliquot of CS stock from each storage condition was thawed and colony forming ability determined by spreading on agar plates as was done for the freshly prepared solution. Bacterial viability was maintained only in the stocks stored at -80°C (Figure 2.5 A). Significant loss of viability was observed in the CS stocks at both one and six weeks following storage at -20 and 4°C (Figure 2.5 A). Similar results were obtained when CS was stored in 5% and 10% glycerol buffer with the colony forming ability maintaining 100% of original capability after cryopreservation at -80°C for 6 weeks (data not shown). Additional CS stocks stored in 15% glycerol/PBS at -80°C were kept for up to six months with bacterial viability being tested periodically. After 6- months of storage at -80 °C, bacterial viability was maintained at 99.5% of the freshly prepared CS (Figure 2.5 B).
Figure 2.5. Cecal slurry bacteria maintain bioactivity following long-term storage at -80°C. Cecal contents were prepared in 15% glycerol and CS samples were stored at -80, -20, and 4°C. Colony formation unit (CFU) was compared one and six weeks later for all storage conditions (A) and up to 6-months later for samples stored at -80°C (B). Data represent the mean ± SD. Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.

2.4.3 CS-induced sepsis reproduces age-dependent sepsis severity and mortality

To test the efficacy of stored CS stocks at inducing sepsis, three different age groups of mice were intraperitoneally injected with standardized doses of CS and survival monitored for at least 10 days. As shown in Figure 2.6 A, 100μL of CS was non-lethal to young and middle-aged mice but caused 50% lethality in aged mice. By increasing the dose of CS from 100μL to 150μL, survival in aged mice was reduced to 38%, survival in middle-aged mice was reduced to 67%, and young mice maintained 100% survival (Figure 2.6 B).
Figure 2.6. CS-induced sepsis reproduces age-dependent mortality. Young, middle-aged, and aged C57BL/6 mice were injected with either (A) 100µL or (B) 150µL CS and survival monitored for eight days. Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.

2.4.4 The severity of CS-induced bacteremia is associated with mortality

To assess the degree of bacteremia in CS-injected mice, blood was collected from the tail vein of mice and bacterial colony formation units assessed by spreading onto agar plates. Blood was collected at 12, 24, and/or 48h and the timepoint with the highest CFU was used. After monitoring survival for 10-15 days, blood CFU was compared between survivors and non-survivors. Circulating bacteria CFU correlated with mortality in various ages of mice injected with 100µL of CS (Figure 2.7 A) or 200µL of CS (Figure 2.7 B).
Figure 2.7. Amount of circulating bacteria correlates with mortality. (A) Blood was taken from the tail vein of young, middle-aged, and aged mice 12, 24, or 48h after injection with 150µL CS and colony formation units (CFU) assessed by spreading on agar plates. After fifteen days, data from mice which survived or died were separated into two groups and CFU compared. (B) Blood was taken from the tail vein of young mice 24h after injection with 200 or 400µL CS and CFU assessed by spreading on agar plates. After ten days, data from mice which survived or died were separated into two groups and CFU compared. Each symbol represents data from a single animal. Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.

2.4.5 CS-induced death is not mediated by endotoxemia

To eliminate the possibility that death due to CS-induced sepsis occurs by endotoxic shock from bolus bacterial injection, an experiment was performed in which CS was heat-inactivated to kill bacteria and then injected to mice (Figure 2.8). The same volume of vehicle (15% glycerol) and untreated CS were injected in parallel. Vehicle injection induced a very mild acute drop in body temperature of approximately 1.5°C which returned to baseline within 12h similar to our previous observation using saline as a vehicle (Saito et al 2003). Both heat-inactivated CS and untreated CS induced profound acute hypothermia with body
temperature falling to 32°C within 3h after injection. Hypothermia was sustained at 32°C in heat inactivated-CS injected mice for 24h at which point the mice began to normalize and all survived, while the untreated CS-injected mice continued to exhibit profound and worsening hypothermia and eventually died. These results suggest that the CS model of sepsis induces non-lethal temporal endotoxemia, but that prolonged illness and death is due to infection.

![Graph showing body temperature over time after CS injection](image)

**Figure 2.8.** Injection of heat-inactivated cecal slurry (CS) induces endotoxemia but not mortality. Mice (13-month-old) were injected with 200µL of untreated CS (in 15% glycerol/PBS), heat-inactivated (72°C for 15 minutes) CS, or vehicle (15% glycerol), and the rectal body temperatures monitored for several days. CS injected mice died within 48 hours, while no mortality was observed in other groups. Data represent the mean ± standard deviation, n=3 for each group. *Figure adapted from Starr, Steele et al. 2014 used under international license CC BY 4.0.*
2.5 Discussion

While performing small animal surgeries or harvesting organs from mice over the last several years (Okamura et al 2012; Saito et al 2003; Starr et al 2012; Starr et al 2009; Starr et al 2010; Starr et al 2011), our group noticed that mice can have highly variable cecum shape and size, and we continuously hypothesized that this variability may affect the result of CLP-induced sepsis. Due to these observations we felt that CLP may not always be an appropriate animal model for inducing experimental sepsis. While others have acknowledged potential problems of the CLP model regarding variability in technique (Dejager et al 2011; Sam et al 1997; Buras et al 2005; Zanotti-Cavazzoni & Goldfarb 2009; Rittirsch et al 2009), variability in animals used for experiments has been largely ignored. Certainly the benefits of using the CLP model may outweigh the limitations compared to other experimental models of sepsis (Dejager et al 2011); however, several characteristics of the animals used in each study design should be carefully considered before assuming that CLP is the ideal model for every experiment. We have shown that the size and shape of the cecum, and/or the nature of cecal contents can be significantly altered by aging or in certain disease conditions (e.g. insufficient digestion in chronic pancreatitis or excessive water consumption in diabetes mellitus). Under these conditions, results from CLP-induced sepsis would be completely misinterpreted, and use of the CS model is a good alternative. For example, CLP performed to compare drug efficacy in mice of the same strain and body size where a subset of the mice receive a drug and another subset do not would be a perfectly appropriate CLP-experiment. While comparing the effects of CLP-sepsis in mice of significantly different body/cecum size (e.g. juvenile vs mature adult, wild-type vs some transgenics), or with significantly different diets or water consumption rates could be problematic due to the nature of the CLP technique.

As aging is one of our research group’s primary interests, we took additional effort to characterize the cecum and cecal contents from young versus aged mice. While we did not observe any statistically significant differences in whole cecum weight, or cecal content weight and wet/dry ratio between the ages
of mice used in this study (4-6 month and 24-26 month), we did notice considerable differences in cecum shape. As can be seen in Figures 2.2 and 2.3, cecum shape can be highly variable, particularly at the distal end (where ligation typically occurs). This variability can alter CLP-sepsis induction since ligation is performed at a designated length, and the shape of the cecum at this location may vary from animal to animal. Our data suggest that if appropriate surgical technique is applied (taking into account variable size and shape of cecum), CLP-induced sepsis likely elicits a similar bacterial infection in young and aged mice. Indeed we and others have shown an age-associated increase in mortality after CLP-induced sepsis (Saito et al 2003; Turnbull et al 2003), similar to the current CS study. However, due to sensitivity of aged mice to surgical manipulation (Rosczyk et al 2008), CLP may cause other significant responses in addition to the effect of infection. After performing CLP on aged mice, we have also noted multiple times that the large epididymal fat pads of aged mice can adhere to the cecal puncture injury and contain the source of infection which would abrogate CLP-induced sepsis. In our experience this occurred when CLP was performed on older ages of mice (10-26 months of age) with more intra-abdominal adipose tissue, but not in lean young mice (2-4 months of age) with minimal amounts of abdominal fat. This observation is similar to what physicians have observed clinically when the omentum adheres to sites of injury within the abdomen (Ambroze et al 1991). Though ideal for survival, this occurrence causes a high degree in variability of response depending on whether and when the fat adhered to the cecal puncture in experimental animals.

In addition to other advantages, the use of the CS model is also valuable in experiments which require large numbers of animals. Experimental sepsis can be easily induced in a large number of animals in a short period of time by CS injection, as opposed to CLP which requires a significant amount of time for anesthesia and surgery on each animal. Likewise, another benefit of the CS model is that surgery is not required, thus potential differences among animal groups in response to surgery or wound healing would be alleviated.
Also important to note, especially in respect to this dissertation work, is that the CS injection model of polymicrobial sepsis is more appropriate when the experimental question centers on evaluating the post-sepsis condition. In the CLP model, cecal ligation is performed in animals given sepsis, however the non-sepsis control animals receive a sham operation without a cecal ligation. Therefore, in long-term studies on sepsis survivors, the necrotic cecum likely stimulates a proinflammatory state, that may be responsible for any observed phenotype, as opposed to sepsis itself. Therefore, we believe the CS injection model is most applicable for these types of studies.

Despite some reports indicating the contrary (Cross *et al* 1993), CS is an infectious model. Other groups have supported this notion with evidence of bacterial colonization, abscess formation, systemic inflammation, and spleen alterations characteristic of infection (Wynn *et al* 2007). We further confirmed this using our new CS preparation protocol since analyses of bacteria in the blood in our present study show a strong correlation with mortality. Further, levels of circulating bacteria which exceed the amount of bacteria administered provide evidence of replication. Heat-inactivation of a lethal dose of CS resulted in no mortality suggesting that CS-mediated death results from the infectious component of CS rather than endotoxic shock due to bolus injection. Studies in rats also showed similarity of the CS injection model with human sepsis in that it induces such physiological changes as hypotension, elevated lactate, leucopenia and leukocytosis, and formation of abdominal abscesses (Lang *et al* 1983; Sam *et al* 1997).

In the preparation of our CS stocks for injection, the cecal contents were suspended in PBS buffer with a final concentration of 15% glycerol before cryopreservation. We used 15% glycerol because it is commonly used for cryopreservation of bacteria (Maniatis *et al* 1982). Fecal transplantation studies show 10% glycerol in saline as a suitable suspension buffer for cryopreservation and future administration of fecal matter to patients without loss of efficacy in patient outcome (Hamilton *et al* 2012). Recently, we also confirmed that CS stocks can be stored in 5 or 10% glycerol without loss of viability for at least 6
weeks, suggesting that suspending cecal contents in glycerol to a final concentration of at least 5% may also be suitable for CS cryopreservation; however, we have not tested survival curves with these preparations.

A limitation of this study is that we did not fully characterize the bacteria in the CS stocks. We do not know what proportion of aerobic or anaerobic bacteria can survive cryopreservation thus the bacteria remaining in the CS stock after freezing and thawing may not be entirely representative of that derived from freshly injected CS or CLP. However, this does not differ from current CS or CLP studies in which the bacterial flora of mice used is not routinely analyzed and can vary from strain to strain and by vendor (Hufeldt et al 2010; Campbell et al 2012; Hildebrand et al 2013). Preparation of CS with our new protocol induces polymicrobial sepsis with organisms endogenous to the host and causes both acute endotoxemia and prolonged bacteremia with the latter being primarily responsible for mortality.

### 2.6 Conclusions

This new cecal slurry (CS) preparation protocol using glycerol/PBS is durable for freezing preservation at -80°C without loss of bacterial viability, allowing us to perform polymicrobial sepsis experiments more conveniently and with higher reproducibility than conventional methods. Injection of mice with cryopreserved CS can reproducibly induce both mild and severe sepsis with mortality rates dependent on both injection dose and animal age. Further, CS-induced mortality is due to bacterial infection, not acute endotoxic shock since (1) heat-treatment of an otherwise lethal dose of CS did not kill the mice, and (2) there was a strong correlation between mortality and blood bacteria counts.
CHAPTER 3

Development of a Late-Intervention ICU-Like Resuscitation Model of Sepsis
Which Results in High Survival

3.1 Abstract

Current animal models of sepsis often incorporate antibiotics to be consistent with clinical standards for treatment of patients in the ICU. However, such experimental intervention is commonly initiated very early after infectious insult, which likely blunts the progression of systemic inflammation and downstream pathology. The objective of this study was to establish an animal model of sepsis with delayed therapeutic intervention, allowing a longer disease course and downstream pathology, but still resulting in a high survival rate. Severe lethal abdominal infection was initiated in young adult (17-18 week-old) C57BL/6 mice by cecal slurry (CS) injection. When initiated early (1 or 6 hours post-CS injection), antibiotic treatment (imipenem, 1.5mg/mouse i.p., twice/day for 5 days) rescued the majority of mice; however, few of these mice showed evidence of bacteremia, cytokinemia, or organ injury. When antibiotic treatment was delayed until late time-points (12 or 24 hours post-CS injection) the majority of animals did not survive beyond 48 hours. When fluid resuscitation (physiological saline, s.c.) was performed in combination with antibiotic treatment beginning at these late time-points, the majority of mice survived (75%) and showed bacteremia, cytokinemia, organ dysfunction, and prolonged body weight loss (<90% for 4 weeks). We recommend that this new repeated combination treatment with antibiotics and fluid resuscitation be initiated at a late time point after bacteremia becomes evident because this model more closely mimics the downstream pathological characteristics of severe clinical sepsis yet maintains a high survival rate. This model would be advantageous for studies on severe sepsis and post intensive care illness.
3.2 Introduction

As the sepsis incidence rate continues to grow and the mortality rate in patients with sepsis and septic shock remains high, the need for a highly translational animal model has become of upmost importance. In recent years, the applicability of animal models for sepsis studies has come under question, spurred by the Seok et al. (2013) report that genomic responses to inflammatory diseases show little to no correlation among mice and humans. This study was highly publicized and sparked unfortunate criticism of biomedical research despite having many study limitations such as disregard of sex, age, strain, or disease severity in their data analysis (Osuchowski et al. 2014). Takao et al. recently reported that after reanalyzing the same data sets taking into account disease conditions and utilizing more conventional statistical methods the conclusion was reversed: gene expression patterns are highly similar among mice and humans (Takao & Miyakawa 2015). Murine models of sepsis are in fact powerful tools for biomedical research, but come with an urgent need to understand the various strengths and weaknesses of different models which must be weighed in the context of the research question being posed (Deitch 2005). The choice of model is a critical decision that heavily influences the relevance of the experimental outcomes in respect to clinical translation ability (Deitch 1998).

To more closely mimic the clinical situation, antibiotic therapy is often included in infectious models (e.g. Turnbull et al. 2003; Newcomb et al. 1998; Xiao & Remick 2005; Marques et al. 2013); however the timing of therapeutic intervention is largely inconsistent due to many unresolved questions in the field regarding how animal models relate to clinical sepsis. Most of these studies administer antibiotics immediately or within a few hours after infectious insult. The time-course of sepsis is accelerated in animals compared to patients, making early intervention with antibiotic therapy a plausible therapeutic strategy, but patients are seldom treated in this narrow window (Turnbull et al. 2004).

We hypothesized that therapeutic intervention initiated prior to the development of bacteremia would halt the progression from local to systemic
infection, thus preventing the development of severe sepsis and downstream pathology. To test this hypothesis, we first assessed the kinetics of bacteremia following infectious insult in adult mice using the cecal slurry model of abdominal sepsis. Further, we methodically determined the most appropriate timing and combination of therapies which when initiated at late time-points allows the progression of local to systemic infection, maintains sepsis pathophysiology, and still results in high survival. Here we present a new repeated combination treatment procedure initiated at a late time point which would be useful for studies on severe sepsis and post sepsis dysfunctions.

3.3 Experimental approach

The objective of the first experiment was to determine the kinetics of bacteremia development following cecal slurry (CS) injection, animals were administered a lethal dose (i.p.) and blood from the tail vein was cultured 1, 6, and 12 hours later. Next, we began our studies on therapeutic intervention. For antibiotic treatment, we used imipenem as it is a broad-spectrum antibiotic with potent capabilities for treating infection and is widely used in animal models of sepsis (Newcomb et al 1998; Coopersmith et al 2003; Marques et al 2013). Imipenem (IPM) was reconstituted in sterile physiological saline for a final concentration of 0.005mg/mL. The dose of IPM equivalent to the maximum dose administered to patients in hospitals was determined to be 1.5 mg. Assuming young lean mice have 2-mL of blood, the final concentration of IPM in the blood (0.75 mg/mL) was used for in vitro bacteria culture and confirmed that CS bacteria (up to 500 CFU/mL) were unable to grow in the presence of IPM, even at 100-times diluted concentrations (Figure 3.1). IPM was aliquoted and frozen at -20°C for up to 1 week (under these storage conditions IPM maintained 95% efficacy).
Figure 3.1. Imipenem prevents the growth of cecal slurry-containing bacteria at physiological concentrations. The growth of cecal slurry bacteria in liquid culture was assessed by optical density (OD_{600}) measurement 24 hours after inoculation of either 200 or 500 CFU in the absence (0mg/mL; negative control) or presence (0.75, 0.075, and 0.0075 mg/mL) of imipenem (IPM), where 0.75 mg/mL represents the approximate concentration in the blood. *Figure adapted from supplemental data shown in Steele et al. 2017.*

Imipenem was administered (1.5 mg per mouse) beginning 1, 6, and 12 hours after CS injection. The early intervention groups received another treatment 12 hours post-CS injection, and all groups then received treatments every 12 hours for 5 days or until death. Survival, body weight, and body temperature were monitored for at least 10 days.

In some experiments, fluid resuscitation (700µL, physiological saline, s.c.) was administered alone or in addition to the 300µL of antibiotics beginning at 12 or 24 hours after CS injection. Antibiotics and fluids were administered twice daily. Antibiotic therapy was continued for 5 days, and fluid resuscitation was
continued until body temperature recovered to at least 35.0°C. Further details are given in Section 7.6.

The bacterial load of animals injected with CS was evaluated before and during therapeutic intervention by culturing small blood samples obtained from micropuncture of the tail vein. A portion of this blood sample was also cultured on agar plates containing antibiotics (2 mg/mL IPM), and the remaining half of the blood sample was spread on plates without antibiotics. Plates were incubated at 37°C for 24 ± 2 hours, colonies were counted, and CFU was calculated. Further details are provided in Section 7.5.

To assess severity of sepsis in relation to timing of therapeutic intervention, cytokinemia and markers of organ injury were evaluated. For this experiment, animals were injected with either CS or vehicle (10% glycerol-PBS). The animals which received CS injection were subdivided into four groups: non-resuscitated, 1h antibiotics, 6h antibiotics, and 12h antibiotics with fluid resuscitation. All animals were euthanized 24 hours after CS or vehicle injection at which time plasma samples were obtained and stored at -80°C until biochemical assay were performed. Interleukin-6 (IL-6), IL-10, IL-1β, and TNF-α levels were quantified (further details are given in Section 7.7). Lung injury was evaluated through histological assessment and blinded scoring, and liver injury was evaluated by quantification of alanine aminotransferase (ALT) using a commercially available kit (further details in Section 7.8).

3.4 Results

3.4.1 The majority of animals cannot be rescued by delayed antibiotic treatment.

First, we determined the kinetics of bacteremia in adult mice after CS-mediated severe infection. Severe infection was initiated by administration of a minimum lethal dose (LD100) of CS (500µL) and no therapeutic intervention was performed. Circulating bacteria was assessed 1, 6, and 12 hours after CS injection. Of the 8 animals assessed, only 2 (25%) had circulating bacteria by 1 hour, which increased to 4 (50%) by 6 hours, whereas all 8 (100%) animals had
bacteremia by the 12-hour time-point (Figure 3.2 A). Based on these data, we defined “early intervention” time-points as ≤6 hours after CS injection at which time bacteremia is not always confirmed, and alternatively “late intervention” time-points as ≥12 hours after CS injection when 100% of animals have circulating bacteria.

Next, we investigated whether mice can be rescued from lethal sepsis if antibiotic therapy is initiated after blood bacteria became detectable in all animals. The potent broad-spectrum antibiotic imipenem (1.5mg/mouse i.p.) was administered to mice beginning at late time-points (12 and 24 hours after CS injection) and the treatment was repeated twice daily for 5 days. As a comparison, some mice also received antibiotic treatment beginning at early time-points (1 and 6 hours after CS injection). A significant improvement in survival (80%, p<0.05 compared with no antibiotics group) was observed when antibiotic treatment was begun 1 hour after CS injection, and the majority of animals (60%) were also rescued when antibiotics were initiated at the 6-hour time-point. However, only 33% (2 of 6) of animals were rescued when initiation of antibiotic treatment was delayed to 12 hours, and no animals survived when delayed further to 24 hours (n=5; Figure 3.2 B). Important to note, the blood bacteria of moribund mice showed no resistance to the antibiotic. Early intervention (starting at 1 and 6 hours) resulted in rapid recovery of body temperature (35.7± 2.3, 36.0 ± 1.9°C respectively) within 48 hours, whereas mice with late intervention developed sustained hypothermia (30.4±2.7°C at 48 hours) indicating that late intervention allows for a prolonged disease time-course (Figure 3.2 C). Taken together, bacteremia does not develop in all mice until 12 hours after CS injection and therefore late therapeutic intervention should be initiated at this time or later. However, antibiotic treatment alone cannot rescue the majority of mice if delayed until these late time-points.
Figure 3.2 Survival rate and disease severity correlate with time of intervention after CS injection. All mice were given a minimum lethal dose of cecal slurry (CS). (A) Blood bacteria load was assessed 1, 6, and 12 hours after CS injection in animals (n=8) which did not receive therapeutic intervention. (B-C) In another experiment, animals received antibiotic treatment (imipenem, IPM; 1.5mg i.p.) beginning 1, 6, 12, or 24 hours after CS injection. Antibiotics were continued twice daily for five days (n=5-6 per group). (B) Survival (star notates $p<0.05$ compared to no-antibiotics group) and (C) body temperature were monitored. Data represent mean ± standard deviation. Figure adapted from Steele et al. 2017.
3.4.2 Late therapeutic intervention with a combination of antibiotics and fluid resuscitation rescues the majority of animals

We aimed to elucidate a therapeutic protocol which could be initiated after development of bacteremia and still achieve a high survival rate. Antibiotic treatment alone (i.p.), fluid resuscitation alone (s.c.), or a combination of both were administered to septic mice beginning 12 hours after CS injection (500 μL) and continued twice daily for 5 days. Without therapeutic intervention, 100% (5 out of 5) mortality was observed by 48 hours confirming our earlier results shown in Figure 3.2 B. Neither antibiotic therapy nor fluid resuscitation alone could rescue a significant number of animals (2 out of 7 and 1 out of 8, respectively). Conversely, combination treatment with antibiotics and fluid resuscitation when initiated 12 hours after CS injection resulted in 75% (6 out of 8) survival rate (Figure 3.3 A). Late intervention with this combination therapeutic strategy resulted in significantly increased body temperature 12 hours after the first treatment (i.e. 24 hours after CS injection; p<0.01 compared to no-intervention group) although body temperature did not fully recover until 48 hours after CS injection (36 hours after therapeutic intervention; 36.1±1.3°C; Figure 3.3 B). Assessment of blood bacteria load 12 hours after CS injection confirmed that bacteremia had developed in all mice before therapeutic treatment was initiated. After 3 treatments with antibiotics and fluid resuscitation (administered every 12 hours), bacteria load was significantly reduced and was completely resolved in all animals after 7 treatments (96 hours after CS injection; Figure 3.3 C).

Further, because mice with sepsis tend to develop hypoglycemia, we tested the effects of glucose control by administering 1-2mg of glucose in the resuscitation fluid to animals with glucose levels below 75mg/dL. Although plasma glucose level transiently increased, hypoglycemia persisted and survival rate was not improved by inclusion of this treatment (data not shown).
Figure 3.3  Late intervention with antibiotics and fluid resuscitation rescues the majority of mice from otherwise lethal sepsis. All animals were given a lethal dose of cecal slurry (CS). Mice received antibiotic treatment alone (imipenem, IPM; 1.5mg i.p.), fluid resuscitation alone (1mL physiological saline, s.c.), or a combination treatment of antibiotics and fluid resuscitation (n=5-9 per group) beginning 12 or 24 hours after CS injection. Therapeutic treatment was continued twice daily for five days. (A) Survival and (B) body temperature were monitored for multiple days. Data is represented as mean ± standard deviation. (C) Circulating bacteria load was assessed in the combination treatment groups by culturing blood obtained by micropuncture of the tail vein immediately before the first therapeutic treatment (12h), after 3 treatments (48h) and after 7 treatments (96h). Symbols * and ** represent $p<0.05$ and $p<0.001$ respectively compared to no intervention group; ††† represents $p<0.001$ respectively compared to bacteria load before intervention (i.e. 12 and 24h). Figure adapted from Steele et al. 2017.
We hypothesized that treating hypothermia using mild cage warming may further improve survival. Therefore, an experiment was conducted in which all animals were given sepsis and were resuscitated beginning at 12h as in Figure 3.3, and cages were either kept at room temperature, or were placed with half of the cage bottom on heating pads so that the bedding was 32-33°C throughout the resuscitation time-course. Such mild cage warming resulted in successful improvement in body temperature compared to animals which received only the antibiotics and fluid resuscitation (34.1 ± 0.7 vs. 29.4 ± 1.6, p<0.001; Figure 3.4 A), but did not improve survival (56% vs. 67%; Figure 3.4 B).

**Figure 3.4.** Mild temperature regulation did not further improve survival in mice which received antibiotic and fluid resuscitation. Middle-aged mice (16 months old) were all administered a lethal dose of CS and received therapeutic intervention with the antibiotic imipenem (IPM) and fluids beginning 12h later. The cages of one group (n=9 mice) were positioned half-on heating pads at this time so that the bedding was mildly warm (between 32 and 33°C), and the remaining mice (n=9) were kept only at room temperature (21-23°C). (A) Rectal body temperature and (B) survival were monitored. * notates p < 0.05, *** notates p < 0.001.
To determine whether high survival is still achievable if therapeutic intervention was postponed even further, combination treatment with antibiotic and fluid resuscitation was delayed until 24h after CS injection. This therapeutic timeline resulted in 50% 10-day survival rate (Figure 3.5 A). The survivors of this delayed intervention exhibited prolonged hypothermia for 48h post-CS injection (Figure 3.5 B). Surviving animals recovered body temperature (36.5±0.8°C) 96h after CS injection (72h after therapeutic intervention). Bacteremia was confirmed at 24h after CS injection and bacteria load was reduced after 3 treatments with therapeutics (72h after CS injection) and was resolved after 7 treatments (108h after CS injection, Figure 3.5 C), similar to the trend observed in the animals that were resuscitated beginning at 12h post-CS injection.
Figure 3.5 Further delay in therapeutic intervention with antibiotics and fluid resuscitation to 24 hours post-CS injection results in 50% survival. All animals were given a lethal dose of cecal slurry (CS). Mice either received no further treatment (n=5), or were administered the therapeutic resuscitation protocol (antibiotic treatment and fluid resuscitation twice daily; n=6), beginning 24h after CS injection and continued twice daily through day 5. (A) Survival and (B) body temperature were monitored for multiple days. Data is represented as mean ± standard deviation. (C) Circulating bacteria load was assessed in the resuscitated animals immediately before therapeutic intervention, after 3 treatments, and after 7 treatments. * and *** represent p<0.05 and p<0.001 respectively compared to no intervention group; † represents p<0.05 compared to bacteria load before intervention. Figure adapted from Steele et al. 2017.
3.4.3 Delayed, but not early, therapeutic intervention allows for the development of cytokinemia, organ dysfunction, and sustained body weight loss

Next we aimed to determine if delayed therapeutic intervention resulted in clinically-relevant characteristics of severe sepsis. Severe infection was initiated by CS injection and animals received either early (≤6 hours) intervention with antibiotics or late (≥12 hours) intervention with combination treatment of antibiotics and fluid resuscitation. Plasma samples were prepared at the time of euthanasia (24 hours after CS injection) and interleukin-6 (IL-6), interleukin 1β (IL-1β), and tumor necrosis factor α (TNFα) levels were assayed. As shown in Figure 3.6, non-resuscitated animals had significantly higher levels of all three proinflammatory cytokines 24 hours after CS injection (the time at which half of animals could still be rescued using repeated antibiotic and fluid treatment) compared to non-sepsis controls (p<0.05). Although significance was not achieved, on average the 12h resuscitated group had elevated cytokine levels compared to non-sepsis controls (18,028 pg/mL vs. nondetectable IL-6 levels, 46.1 vs 18.4 pg/mL IL-1β levels, and 67.1 vs. 5.0 pg/mL TNFα levels). Similarly, the 12h resuscitated group had higher cytokine levels on average compared to that of the early (≤6 hours) resuscitated groups. A similar trend was observed without statistical significance when the anti-inflammatory cytokine interleukin 10 (IL-10) levels were compared among the groups.
Figure 3.6 Early, but not late, therapeutic intervention blunts CS-induced cytokinemia. Severe infection was induced by cecal slurry (CS) injection and mice were divided into groups (n=4-8). Mice received antibiotic treatment (imipenem, 1.5 mg/mouse, i.p.) beginning 1 or 6 hours after CS injection, or combination treatment with antibiotics and fluid resuscitation (physiological saline, s.c.) beginning 12 hours later. A group of animals (n=4) received vehicle injection (10% glycerol-PBS) for comparison (non-sepsis). Plasma samples obtained 24 hours after CS injection were subjected to (A) IL-6, (B) IL-1β, (C) TNFα, and (D) IL-10 quantification. * indicates $p<0.05$ by one-way ANOVA. Figure adapted from Steele et al. 2017.
In order to assess potential long-term effects of delaying therapeutic intervention, the body weight of each mouse was monitored for 14 days after CS injection. Mice which received early intervention (starting at 1h or 6h) experienced rapid recovery of body weight (> 95% or >90% of original body weight by 7 days, respectively), whereas delayed intervention (starting at 12h or 24h) resulted in long term body weight depression (<85% original body weight by day 7, and <90% by day 14, p<0.05 early time-points vs late time-points; Figure 3.7 A). In another experiment under the same sepsis and therapeutic conditions, we confirmed that the sepsis-induced reduction in body weight (<90% original) persisted for at least 4 weeks (Figure 3.7 B).

**Figure 3.7** Late, but not early, therapeutic intervention results in prolonged reduction in body weight. Severe infection was induced by cecal slurry (CS) injection and mice were divided into groups (n=4-8). Mice received antibiotic treatment (imipenem, 1.5 mg/mouse, i.p.) beginning 1 or 6 hours after CS injection, or combination treatment with antibiotics and fluid resuscitation (physiological saline, s.c.) beginning 12 hours later. (A) Body weight was monitored for 14 days after CS injection (n=5-9). (B) In a separate experiment, the body weight of animals resuscitated with antibiotics and fluid resuscitation beginning 12 hours after CS injection was monitored up to 30 days post-CS injection (n=7). †† indicates p<0.01 at all time-points compared to baseline (paired student’s t-test). *Figure adapted from Steele et al. 2017.*
Delayed therapeutic intervention also resulted in abscess formation which was identified in approximately half of the animals two weeks after sepsis induction (Figure 3.8).

**Figure 3.8** Abscesses were detected in surviving mice two weeks after sepsis induction by cecal slurry with late therapeutic intervention. A small abscess on the liver surface (A) and a larger abscess on the visceral adipose tissue (B) are shown as examples. *Figure adapted from supplementary data shown in Steele et al. 2017.*

To evaluate the effect of timing of therapeutic intervention on sepsis-induced organ injury, lung injury of animals euthanized 24 hours after CS injection was histologically examined. As shown in Figure 3.9, H&E staining of tissue sections revealed that late resuscitation, started ≥12 hours after CS injection, resulted in pathological abnormalities including infiltration of inflammatory cells, marked edema in the interstitial space, and thickened alveolar walls. The average composite lung injury score of 12-hour intervention group (7.5) was equivalent to the non-intervention group’s average score (7.8), both being much higher than the control group (0.8). Conversely, animals which received early intervention (1 or 6 hours after CS injection) showed few markers of inflammation (average composite score of 2.2). The inflammatory scores of the 12 hour and non-resuscitated groups were significantly higher compared to the early resuscitated groups (1 and 6 hours) (p<0.001).
Figure 3.9 Early, but not late, therapeutic intervention prevents sepsis-induced lung injury. Mice received cecal slurry (CS; n=14) or vehicle (10% glycerol; n=4) injection. CS-injected mice were divided among groups: non-resuscitated, 1h antibiotics, 6h antibiotics, or 12h combination treatment with antibiotics and fluid resuscitation (n=3-4 / group). All animals were euthanized 24h after CS or vehicle injection. (A-E) Representative images of lung tissue sections stained with H&E (400X, scale bar represents 50 µm) are shown. (F) Histopathological scoring (n=3-4 per group). * represents p<0.05. Figure adapted from Steele et al. 2017.
To further assess the effect of delaying therapeutic intervention on organ injury, plasma alanine aminotransferase (ALT), a commonly used marker of liver damage, was quantified in the samples obtained 24 hours after CS or vehicle injection. As displayed in Figure 3.10, the ALT levels of animals which received delayed intervention (12 hours after CS injection) was comparable to those which did not receive therapeutics (63.2 and 69.9 ng/µL, respectively) (p<0.05) which were both statistically higher than the vehicle injected non-sepsis controls (14.2 ng/µL). ALT levels in animals which received early therapeutic intervention (1 and 6 hours after CS injection) showed moderate but non-significant ALT increases compared to controls. Together, these data suggest that delaying intervention to late time-points allows pathological characteristics of severe sepsis to develop, which were not observed in early intervention groups.

![Figure 3.10](image-url)

**Figure 3.10** **Delayed therapeutic intervention results in heightened plasma ALT levels.** Animals were divided among treatment groups as described in Figure 3.9. Plasma samples obtained 24 hours after CS injection were subjected to ALT quantification by colorimetric assay (n=3-4 per group). * indicates p<0.05. *Figure adapted from Steele et al. 2017.*
3.5 Discussion

To mimic the clinical situation, antibiotics are often included in animal models of sepsis. However, most of the studies using these animal models intervene either immediately or within a few hours after infectious insult. Since previous studies have not evaluated the effect of the timing of therapeutic intervention in relation to timing of bacteremia after induction of severe infection in animal models, there has been a question on whether therapeutic intervention is conducted in a manner to maximize survival without allowing severe sepsis to develop. We hypothesized that intervention, if initiated before the development of bacteremia, would halt the progression from local to systemic infection and block downstream pathology otherwise characteristic of severe sepsis. On the other hand, we hypothesized that delaying intervention until after bacteria is detectable in circulation would allow propagation of the inflammatory cascade and organ damage, but still result in a high survival rate if the therapeutic strategy is aggressive.

For this study, the cecal slurry (CS) injection model was ideal due to being highly time efficient, allowing the induction of sepsis in a large number of animals within a short period of time (Gentile et al 2014; Shrum et al 2014; Sam et al 1997). Additionally, it is highly reproducible using our new CS preparation protocol (Starr, Steele et al 2014; Starr, Steele et al 2017). Using this model, we showed that bacteria are not always detectable in the circulation of animals until 12 hours after injection with a lethal dose of CS. Only 25% of animals were positive for circulating bacteria 1 hour after CS injection, and even 6 hours after CS injection, 50% of animals remained negative for circulating bacteria.

Using these data, we attempted to establish an intervention model which is initiated after all animals are positive for bacteria in the circulation (i.e. ≥12h after sepsis induction). Initiating antibiotic therapy prior to this time-point resulted in relatively high survival rates: i.e. intervention at 1 and 6 hours resulted in 80 and 60% survival, respectively, which is in agreement of the study by Gonnert et al. (2011) in which antibiotic therapy initiated 2 hours after CS injection resulted in a 50% survival rate. However, our data indicate that many of these animals did
not develop sepsis at such early time points. When we delayed intervention until late time-points (≥12 hours when bacteremia is apparent in all mice), antibiotic treatment alone could not rescue the majority of animals and >67% died within 4 days. Importantly, the blood bacteria of moribund animals (determined by body temperature less than 32°C for over 24 hours following CS injection) were unable to grow in the presence of IPM when cultured on agar plates containing the antibiotic. This evidence verifies that mortality in this model is not a result of antibiotic resistance of the circulating bacteria.

We then attempted to increase survival rates of animals under late intervention by including fluid resuscitation to reduce hypotension, another lethal component of sepsis. In attempt to mimic the continuous fluid resuscitation state without the use of anesthesia, we conducted fluid resuscitation via subcutaneous injection two times a day. We observed a significant improvement in survival rate (≥75% of animals with otherwise completely lethal sepsis) when antibiotic treatment and fluid resuscitation were given in combination starting 12 hours after sepsis induction. This 75% survival rate by the combined treatment is a striking improvement compared to 29% by antibiotics alone and 13% by fluid resuscitation alone. Our recent study confirmed that this resuscitation protocol can effectively rescue approximately 70% of older mice as well (data not shown). Importantly, when the combined therapeutic intervention was further delayed for a full 24 hours after CS injection, half of the animals could still be rescued using this combination treatment. In these experiments, some of the animals with the most severe bacteremia could be rescued using the combination treatment. This demonstrates the effectiveness of this resuscitation protocol, especially since our group previously showed that without therapeutic intervention, bacteremia following CS injection correlated with 15 day survival (Starr, Steele et al 2014).

In this study, we also provided data demonstrating that animals with late intervention, but not early intervention, develop pathophysiological conditions that are characteristics of sepsis. We found that intervening after bacteria was confirmed in circulation (≥12 hours after CS injection) resulted in the elevation of plasma IL-6, IL-1β, and TNFα levels 24 hours after CS injection when compared
to animals in which therapeutic intervention was initiated at early (1 and 6 hours) time-points. Further, late intervention resulted in marked lung injury, characterized by inflammatory cells, thickened alveolar walls, and edema, as well as liver damage assessed by heightened plasma ALT levels. We also found that surviving animals of the late intervention groups exhibited an inability to recover body weight for four weeks after sepsis. Being that the majority of animals showed no signs of body weight recovery beyond the 1 week time-point, it is highly likely that these mice will have reduced body weight beyond this 4-week time-point. Therefore, we conclude that our experimental sepsis model with late intervention allows for good survival rates with development of severe sepsis, organ injury, and long-term body weight reduction, as opposed to sepsis models with early intervention which tend to blunt pathophysiological processes, and sepsis models without intervention which result in high early mortality.

We predict that this late therapeutic strategy would be highly applicable to other animal models of sepsis, including the widely used CLP model. The CS and CLP models are similar in that they induce severe intra-abdominal infections via exposure to endogenously-derived polymicrobial bacteria. Indeed, many have found that antibiotic administration after CLP significantly improves survival (Newcomb et al 1998; Coopersmith et al 2003; Brown et al 2015; Baker et al 1983), especially in the young (Turnbull et al 2003). When administration of antibiotics was delayed until 12 hours after CLP and given every 12 hours for 5 days, animals with IL-6 levels >14,000 pg/mL at 6 hours were unable to be rescued (Turnbull et al 2004). In our model, the average IL-6 level were higher than the cutoff set by Turnbull et al, (106,445 pg/mL at 24 hours after CS injection). We too found that animals were not able to be rescued using antibiotics alone, however, survival was achieved in half of the animals when given combination treatment, despite having 7-times greater IL-6 levels than the cutoff established by Turnbull et al. Thus, we have reason to believe that if this resuscitation strategy were applied to the CLP model, similar survival rate would be achieved. Important to note, the development of bacteremia in the CLP model is presumably slower than what we observed in the CS model because
the cecal contents may leak more slowly into the peritoneum in the CLP model as opposed to the bolus injection of cecal contents in the CS model. Therefore, the more clinically relevant appropriate time of intervention may need to be determined and therapeutic treatments may need to be continued for more than 5 days.

To apply this late-intervention model to other sepsis models or even different experimental conditions (i.e. different age, strains, or gender), we recommend that the kinetics of bacteremia following infectious insult should first be assessed before deciding the time point for therapeutic intervention. For example, bacteremia was confirmed in all mice just 2 hours following CS injection to 5-7 day-old neonate mice (Wynn et al 2007), indicating that intervention at 12 hours may be too late and thus not effective in this case. It is our opinion that this protocol, combination treatment with antibiotics and fluid resuscitation performed twice daily for multiple days, should be initiated only after bacteremia is confirmed to ensure the development of sepsis and a longer disease course.

Although this late-intervention protocol is multifactorial, we acknowledge that this is not a comprehensive therapeutic strategy. The infection and hypotension aspects of sepsis are targeted therapeutically in this protocol, as they are primary concerns clinically. Although we did not characterize which bacteria grew in the blood cultures or the peritoneum, we confirmed that the bacteria in both the CS stocks and blood bacteria were not resistant to IPM. We also examined the efficacy of glycemic control in our late-intervention model with antibiotic treatment and fluid resuscitation; however it showed no further benefit to survival outcome. In this study we did not include ventilatory support, but predict that it would be beneficial not only for clinical relevance but also for survival. However, we aimed to develop a therapeutic strategy which could be adopted by many laboratory groups, and prolonged murine ventilation is a technically challenging technique which requires specialized equipment and also adds variables (such as prolonged use of anesthesia (Muller-Redetzky et al 2014; Schellekens et al 2015)) to the experiment which is often undesirable, but would be worthwhile to include in future experiments.
3.6 Conclusions

To the best of our knowledge, this is the first study which examined the effect of therapeutic intervention in relation to the kinetics of the development of bacteremia following infectious insult in an animal model of sepsis. Here we elucidate a late-intervention combination treatment protocol in which antibiotic therapy and fluid resuscitation are repeatedly administered and result in significantly improved survival, even after progression from local to systemic infection is achieved, as is profound cytokinemia and organ damage (characteristics of clinical sepsis). As summarized in Figure 3.11, late intervention with combination therapeutics after infectious insult has several advantages over existing sepsis models in which intervention is either not performed or initiated at early time points. We recommend use of this new procedure as it will allow for more clinically relevant studies on severe sepsis and on investigating post intensive care illness.

**Figure 3.11 Early vs. Late therapeutic intervention: A comparison of sepsis outcomes after severe infection in murine models.** Late therapeutic intervention starting 12 hours after severe infection allows development of severe sepsis with organ injury and long-term weight reduction. *Figure adapted from Steele et al. 2017.*
Portions of this chapter were adapted and reprinted by permission from Wolters Kluwer: Steele AM, Starr ME, Saito H (2017). Late Therapeutic Intervention with Antibiotics and Fluid Resuscitation allows for a Prolonged Disease Course with High Survival in a Severe Murine Model of Sepsis. Shock 2017; 47(6):726-734.
CHAPTER 4

Characterization of Muscle Weakness in Sepsis Surviving Mice:
Muscle Quantity vs. Quality

4.1 Abstract

Sepsis is a serious problem among the aging population as both susceptibility and mortality increase dramatically with age. Among survivors, >70% report muscle weakness that significantly impacts activities of daily living for years after hospital discharge. The objective of this study was to adapt our sepsis/resuscitation protocol using late middle-aged animals (16 month-old C57BL/6 male mice) and to then assess muscle strength in the sepsis survivors long after bacteremia was resolved. Further, we sought to assess changes in muscle size during and after sepsis to understand the kinetics of sepsis-induced atrophy. To do this, experimental sepsis was induced in 16-month-old male mice by cecal slurry (CS) injection. Antibiotics and fluid resuscitation were administered beginning 12-hours after CS injection and were continued twice daily for five days to rescue the majority of animals from otherwise lethal sepsis. Changes in body weight and composition were monitored during and after sepsis. Skeletal muscles were weighed and harvested from non-sepsis controls, animals with sepsis (day 4), and after sepsis (day 14). Plasma samples were also obtained at these time-points. During sepsis, mice lost an average of 1.7 grams lean mass by day four ($p<0.01$) which recovered by Day 10 ($p=0.4$). Post-sepsis animals were significantly weaker compared to controls as evaluated by ex vivo specific force analysis of the extensor digitorum longus was ($p=0.001$). Mean cross-sectional area of fiber-types (type I, IIa, IIb, and IIx) in the gastrocnemius were reduced during sepsis (day 4) but were similar among non-sepsis controls and post-sepsis, indicating that atrophy was resolved by day 14. Histological examination of the tibialis anterior and soleus muscles by H&E and ATPase histochemical stain revealed hallmarks of critical care myopathy.
Altogether these data suggest that myopathy, rather than atrophy, is the underlying primary mechanism of muscle weakness in the post-sepsis condition.

4.2 Introduction

As detailed in Section 1.2, sepsis survivors suffer from long-term muscle weakness which significantly impacts their quality of life. Often this results in severe limitations in activities of daily living which cause previously independent individuals to be discharged to nursing care facilities which is further illustrated in Figure 4.1. (Odden et al 2013; Iwashyna et al 2010). This lack of a clinically relevant model of sepsis has been a critical barrier to identification of the mechanisms responsible for sepsis-induced long-term muscle weakness, therefore limiting progress in the field.

Figure 4.1 Sepsis-induced muscle weakness results in the loss of independence. Upon hospital discharge, sepsis survivors were documented as being discharged to a nursing care facility, home with hospice, or home with no care. These data were further categorized by patients who previously had no limitations of activities of daily living before hospitalization for sepsis (A) and those who did have limitations previously to hospitalization (B). Adapted from Battle et al. 2014 used under international license CC BY 4.0.
Further, few studies take into account that sepsis is a disease of the aged, as introduced in Section 1.4. Middle-aged individuals are not only at an increased risk for developing sepsis than the young, they are also more likely to develop post-sepsis dysfunction that persists for years after hospital discharge, possibly due to an already sedentary lifestyle, reduced muscle mass, and lower protein intake (Contrin et al 2013; Rahman et al 2013). The middle-aged, who comprise 26.4% of the American population, are particularly vulnerable to repercussions of sepsis-mediated dysfunction being that they are in their peak salary earning years and sepsis-mediated functional limitations threaten their ability to return to work (Iwashyna et al 2012; Iwashyna et al 2010; Hofhuis et al 2008; Battle et al 2014). Thus, when choosing an animal model of sepsis, age should be a careful consideration for clinical translation.

In the present study we adapted the severe model of murine sepsis with delayed but aggressive ICU-like resuscitation (Steele et al. 2017; Chapter 3) and demonstrated that middle-aged sepsis-surviving mice exhibit significant muscle weakness long after recovering from sepsis pathogenesis (2-weeks after sepsis induction). We found that this long-term muscle weakness is present even after sepsis-induced atrophy is resolved, and when strength was normalized to muscle size, indicating that post-sepsis chronic muscle weakness in the sepsis survivors was due to poor muscle quality, rather than quantity.

4.3 Experimental approach

Cecal slurry (CS) was prepared as described in Section 7.3 and polymicrobial abdominal sepsis was induced by bolus CS injection as described in Section 7.4 in late middle-aged male mice (16-months-old; C57BL/6) using a lethal dose (LD100; 400 µL). Therapeutic intervention (antibiotics and fluid resuscitation) was administered beginning 12 hours after CS injection and continued twice daily for at least 5 days, as established using young animals as described in Chapter 3 (and in methods section Section 7.6). Bacterial load (Section 7.5) was evaluated immediately before therapeutic intervention, again at 48 hours (after 3 treatments) and 96 hours (after 7 treatments) after CS injection.
Body weight and body composition (fat and lean mass) were assessed before, during, and after sepsis using echoMRI technology. Based on trends of lean mass following CS injection observed using echMRI analysis and to better understand changes in muscle size after sepsis induction, groups of animals were euthanized during sepsis (day 4) and post-sepsis (day 14) along with non-sepsis controls, and hind-limb skeletal muscles were weighed. Ex vivo analysis of isometric specific force was used to evaluate muscle strength in sepsis survivors (day 14) compared to non-sepsis controls and resuscitated-only controls (methods described in Section 7.10). Pilot experiments were also conducted to monitor activity as well as strength throughout the sepsis time-course using grip strength and the inverted hanging test (Figure 7.2). Skeletal muscles from animals during and after sepsis were subjected to histological observation by hematoxylin and eosin (H&E) staining. Adenosine triphosphatase (ATPase) histochemistry was also performed using pre-incubation pH 10.2, which results in light staining of slow twitch fibers and dark staining of fast twitch fibers.

4.4 Results

4.4.1 Animals rescued using our sepsis/resuscitation protocol exhibit long-term muscle weakness

We first adapted our severe model of sepsis which includes delayed but aggressive ICU-like therapy to rescue the majority of mice to middle-aged mice. Sepsis was induced by bolus injection of cecal slurry (CS), and animals were randomized to two groups: non-resuscitated group (n=10) or therapeutic intervention group which received antibiotics (imipenem; IPM) and fluid resuscitation initiated at 12-hours after CS injection and continued repeatedly twice daily for at least five days (n=54 over the course of several experiments). Therapeutic intervention rescued 74.1% of animals from otherwise completely lethal (LD100) sepsis ($p<0.01$, Figure 4.2 A). Body temperature was significantly improved within 12-hours of the first administration of antibiotics and fluids (i.e. 24-hours post-CS injection), however, on average animals remained hypothermic
for several days, demonstrating a prolonged disease time-course similar to clinical sepsis (Figure 4.2 B). This is further supported by bacteremia analysis (Figure 4.2 C); all animals developed bacteremia prior to therapeutic intervention, however 3X antibiotic administration was not sufficient for bacterial clearance in over half (53.3%) of animals, but was resolved by the end of the resuscitation time-course (day 4; antibiotics were continued for one more day to prevent expansion of potentially ongoing local infection). Therefore, this delayed but aggressive ICU-like resuscitation animal model of sepsis allows for a longer disease time-course while also achieving a high survival rate.
Figure 4.2 Delayed but aggressive therapeutic intervention rescues the majority of middle-aged animals from otherwise lethal sepsis. Sepsis was induced by bolus injection of cecal slurry (CS, i.p.). A group of animals received no further treatment (n=10), and others received therapeutic intervention beginning 12-hours after CS injection, and continued twice daily for 5 days. Survival (A; total n=54 animals which received CS injection and therapeutic intervention) and body temperature (B) were monitored for 14 days (body temperature data shown through day 4, when temperatures returned to baseline values, n=10 non-resuscitated, n=21 resuscitated). In the group which received therapeutic intervention, circulating bacterial load was assessed immediately before the first therapeutic intervention (i.e. 12 hours after CS injection), and again on days 2, 4, and 14 (C, n=14).
In the next study, animals were euthanized 14-15 days after CS injection along with non-sepsis controls, when animals had been normothermic and clear of bacterial load for ≥10 days. *Ex vivo* muscle function was assessed by force-frequency relationship analysis, normalized to physiological cross-sectional area to measure specific force *(Figure 4.3 A).* Sepsis survivors exhibited a 19.3% decrease in maximal specific force compared to non-sepsis controls *(Figure 4.3 B; 152.87 ± 2.17 vs. 189.51 ± 1.99 kN/m2).* To evaluate the possible influence of antibiotic administration on muscle function, specific force was measured in animals which received vehicle injection (10% glycerol) instead of CS and all resuscitation procedures; no change in muscle function was observed *(p=0.6 vs. non-sepsis controls; 194.89 ± 2.19 kN/m2).* These results demonstrate that this cecal slurry induced sepsis protocol with delayed but aggressive ICU-like resuscitation produces sepsis surviving animals which have significant muscle weakness even long after sepsis itself is resolved, and is not attributable to antibiotic treatment.
Figure 4.3. Animals rescued from lethal sepsis using our resuscitation protocol exhibit long-term muscle weakness. Sepsis was induced by CS injection and animals were resuscitated beginning 12-hours for 5 days and animals were euthanized on day 14. (A) Muscle strength was assessed by \textit{ex vivo} isometric force analysis performed on the EDL of non-sepsis (n=9), resuscitation-only (n=7), and sepsis surviving mice (n=10), and the maximum specific force (200 Hz) was observed (B). Data expressed as means ± SD.
In a small pilot experiment, sepsis was induced in late middle-aged (18-month-old) BALB/c female mice and resuscitation was performed as described beginning 12 hours after CS injection. Strength was evaluated in the sepsis survivors (n=2) and the females showed a similar trend of muscle weakness compared to the non-sepsis controls (n=3) (Figure 4.4) similarly to what was observed in male C57BL/6 mice (Figure 4.4), suggesting that post-sepsis muscle weakness is not sex specific. A large limitation in this experiment, however, is that it was conducted in a different mouse strain as well as different sex, which introduced a large variable. Therefore a similar experiment must be done in female C57BL/6 animals to make conclusive remarks on any influence of sex on this phenomenon.

**Figure 4.4.** Female BALB/c mice develop long-term muscle weakness after sepsis. Sepsis was induced cecal slurry injection and animals were resuscitated beginning 12-hours after CS injection, and continued twice daily for 5 days. Animals were euthanized on day 14 and muscle strength was assessed by *ex vivo* isometric force analysis performed on the extensor digitorum longus (EDL) of non-sepsis control (n=3) and sepsis surviving mice. Data expressed as means ± SD.
4.4.2 Behavior-based assessments of rodent strength were not robust enough to make conclusions on the progression of muscle weakness in sepsis survivors

To evaluate how muscle weakness progressed after sepsis, our initial experiments included analysis of grip strength, a commonly performed method for evaluating strength in rodents, and the inverted hanging test (shown in Figure 7.2). We conducted a small pilot experiment using non-sepsis control animals to evaluate the reproducibility of forelimb grip strength, hindlimb grip strength, and total grip strength, and found that hindlimb strength was the least variable. Therefore, only hindlimb grip strength was used in the subsequent experiment which showed no difference among animals that just recovered from sepsis (day 5 after cecal slurry injection) when compared to baseline or non-sepsis controls, which was also true on day 14 (Figure 4.5 A). On the other hand, both groups improved performance in the inverted hanging test (Figure 4.5 B), which likely is a result of learning with increased exposure to the task.

Figure 4.5. Sepsis-induced muscle weakness is not observable using behavioral assessments of rodent strength. Strength was measured using hindlimb grip strength (A) and inverted hanging test (B). Baseline measurements were recorded for all animals (n=11). Animals were randomized to non-sepsis control (n=4) and sepsis (n=7) groups. Strength was assessed on day 6 (the day after the resuscitation time-course was completed), and again on day 14. Data are expressed as means ± SD.
4.4.3 Sepsis induces long-term changes in activity

As assessed by spontaneous wheel running activity, sepsis survivors (days 6 & 7 after CS injection) are significantly less active compared to baseline (Figure 4.6 \( p<0.05 \)). Although this assessment of activity was promising, there was a concern on the effect of exercise on immune function and overall sepsis pathogenesis, making interpretation of results difficult.

![Figure 4.6](image_url)  
Figure 4.6. Spontaneous wheel running activity is reduced after sepsis. Animals were split to wheel running cages and acclimated for ten days. Spontaneous wheel running was then recorded for five days as the "before" measurement. Animals were randomized to either the sepsis group or non-sepsis control group which received vehicle injection (10% glycerol-PBS) and all therapeutic procedures. Resuscitation was performed for five days, and wheel running was recorded for the two days following completion of the resuscitation time course (days 6 and 7 following CS injection). Data are expressed as means ± SD.
In a small pilot experiment, cage activity was monitored using infra-red motion detectors. Five animals were initially used, however only three animals survived sepsis and could be used for analysis. Animals were split to the activity cages and acclimated for one week. Baseline measurements were recorded for two weeks and were largely consistent (Figure 4.7). Activity was reduced during the week of sepsis induction with resuscitation (week 0) and remained significantly lower compared to baseline for three weeks ($p<0.05$ week 1 after sepsis, $p<0.001$ weeks 2 and 3 after sepsis), demonstrating that sepsis induces long-term reductions in activity.

**Figure 4.7. Sepsis induces long-term reductions in cage activity.** After a one-week acclimation period (data not shown), cage activity was monitored before sepsis induction for two weeks (weeks -2 and -1). Sepsis was induced by cecal slurry injection and therapeutic resuscitation was repeatedly administered for five days (week 0). Activity was monitored for three weeks after recovery from sepsis (weeks 1, 2, and 3). The daily number of beam breaks were averaged for the week, and data are expressed as these means ± SEM. Statistics compare experimental week to the average of pre-sepsis weeks.
We then evaluated percentage of activity occurring during light and dark phases using the cage activity data acquired using infra-red motion detectors. We found that sepsis survivors showed similar percent activity during light phase (29.0%) compared to before sepsis (32.7%), indicating that the sepsis survivors did not have a circadian rhythm shift (Figure 4.8 A). However, using piezoelectric recording of sleep, we found that duration of sleep bouts during the dark phase is significantly increased in sepsis survivors compared to controls (Figure 4.8 B), demonstrating that sleep behavior may be altered in sepsis survivors.

Figure 4.8. Circadian rhythm is unchanged in sepsis survivors but sleep bout length is increased. Animals were acclimated for one week in cages with infra-red motion detectors, and activity was recorded for two weeks (pre-sepsis). Sepsis was induced by cecal slurry injection, therapeutic resuscitation was performed, resulting in three sepsis survivors. Percent activity during light and dark phase was calculated for the pre-sepsis period and the week after sepsis (days 8-13; A). Sleep behavior was analyzed using piezoelectric recording in non-sepsis controls (n=7) and post-sepsis animals (days 8-9 after CS injection; n=9; B). Data are expressed as means ± SEM.
4.4.4 Sepsis-induced atrophy is evident using our sepsis/resuscitation model, and is recovered by day 14 after sepsis induction

To further support this direction, we conducted a series of experiments to demonstrate recovery of muscle mass in our sepsis surviving mice. The body weight of animals in the sepsis group steadily declined through day 4 (average 4.2 ± 0.2 g; 11.6 ± 0.4 %). After recovery from sepsis (by days 4-5) animals no longer lost weight, but did not recover to baseline by the end of the experiment on day 14-15 (8.0 ± 0.8% below baseline weight, Figure 4.9 A). To better understand this sustained body weight loss, fat and lean mass were assessed at baseline and throughout the experimental time-course using EchoMRI technology in non-sepsis controls (n=7) and animals given sepsis (n=11). Animals lost an average of 2.41 ± .01 g of fat mass by day 4, and remained significantly lower than baseline at the end of the experiment (2.09 ± .01 g, p<0.001, Figure 4.9 B). Lean mass, also, decreased by day 4 (1.7 ± 0.1 g compared to baseline) but unlike fat mass, did recover to baseline values by day 14-15 (p=0.4, Figure 4.9 C). These results indicate that fat mass primarily accounts for the sustained body weight reduction in the murine sepsis survivors.
Figure 4.9. Lean mass is reduced during sepsis but recovers by day 14 after cecal slurry (CS) injection. Animals either received no treatment (non-sepsis controls) or CS injection with therapeutic intervention beginning at 12-hours. Body weight was monitored daily during resuscitation time-course, and on days 7, 10, and 14 (A). Fat (B) and lean (C) mass were regularly analyzed using EchoMRI technology (n=7 non-sepsis controls, n=11 sepsis with resuscitation). Data are expressed as means ± SEM. n.s. not significant (statistically).
To more closely evaluate changes in skeletal muscle mass itself at time-points identified by assessing the kinetics of atrophy in our sepsis model shown in Fig. 4.8 B, we euthanized groups of non-sepsis animals, animals with sepsis (day 4), and animals recovered from sepsis (day 14) and measured the wet weight of multiple hind-limb muscles with different metabolic properties from one leg, and muscles from the other leg were preserved for histology (described in methodology section) for fiber-type specific cross-sectional area analysis. The wet weight of glycolytic (tibialis anterior and extensor digitorum longus), and mixed metabolic type (gastrocnemius) muscles (with similar trend in an oxidative muscle, the soleus) were significantly lower in animals with sepsis \( (p<0.01, <0.001, \text{ and } <0.01, \text{ respectively}) \) but were comparable to controls in the post-sepsis group \( (p=0.1, 0.2, \text{ and } 0.1, \text{ respectively}) \), again suggesting that muscle mass is recovered by day 14 after CS injection, approximately 10 days after recovery from sepsis (Figure 4.10).
Figure 4.10. Skeletal muscle wet weight is reduced during sepsis but recovers to control values by day 14 after cecal slurry (CS) injection. Animals either received no treatment (non-sepsis controls) or CS injection with therapeutic intervention beginning at 12-hours. Wet tissue weight of various hindlimb skeletal muscles were measured from control (n=17), sepsis (day 4, n=7), and post-sepsis (day 14, n=12) animals. Data are expressed as means ± SEM. n.s. not significant (statistically).

Finally, cross-sections of skeletal muscles were cut, fluorescently labeled for fiber-type, and fiber-type specific cross-sectional area was quantified as a robust measure of muscle size. Similarly to wet muscle weight, the cross-sectional area for fast-twitch fibers (types IIa, b, and x) as well as slow-twitch fibers (type I) were significantly smaller in the muscles from animals with sepsis, but were comparable to non-sepsis controls in the post-sepsis animals (day 14, Figure 4.11). Together, these data indicate that sepsis surviving mice have significant muscle weakness but is not attributable to atrophy; thus dysfunction within the myofibers must be the predominant contributing factor.
Figure 4.11. Cross-sectional area is reduced in fast-twitch myofibers during sepsis but is recovered to that of controls by day 14. Animals either received no treatment (non-sepsis controls) or CS injection with therapeutic intervention beginning at 12-hours. The medial head of the gastrocnemius was fiber-typed using immunofluorescent labeling of myosin heavy chains for non-sepsis (n=5), sepsis (day 4, n=4), and post-sepsis (day 14, n=5) groups, and cross-sectional area was measured using a semi-automated program. Data are expressed as means ± SEM. n.s. not significant (statistically).
4.4.5 Skeletal muscle of sepsis survivors exhibit hallmarks of critical care myopathy

Neuromuscular diseases are often characterized by sets of histopathological markers, which are sometimes used as a tool for diagnosis. These routine stains used clinically can be insightful for research scientists as tools to similarly “diagnose” animals in our research studies. Thus, we performed adenosine triphosphatase (ATPase) histochemical stain (pre-incubation pH: 10.2) on muscle cross-sections of all three groups (non-sepsis controls, animals during sepsis, and animals recovered from sepsis), and compared these sections with a serial section stained with hematoxylin and eosin (H&E). The mosaic of glycolytic (darkly stained) fibers and oxidative (lightly stained) fibers is comparable among all three groups in both the tibialis anterior and soleus muscles, indicating that fiber-type grouping (characteristic of polyneuropathy and/or neurogenic atrophy) is not evident in the sepsis survivors. However, the sepsis survivors’ muscle is characterized by numerous fibers with pockets of ATPase activity (Figure 4.12, notated by arrows), which is not seen in the controls or animals with sepsis. Serial section with H&E demonstrates that the hole is not due to loss of tissue in the cross-section. These fibers which lack ATPase activity have been reported in patients with sepsis or other acute inflammatory conditions (Wheeler 1982), and is a common feature of critical illness myopathy. These data indicate that our sepsis animal model closely mimics the clinical situation, and further that sepsis survivors have dysfunction within the myofiber which may be responsible for the muscle weakness phenotype.
Figure 4.12 Abnormal pathology is evident in skeletal muscles of murine sepsis survivors. The tibialis anterior and soleus muscles were harvested from non-sepsis controls (A), cecal-slurry injected animals during the resuscitation timecourse (day 4, B), and post-sepsis animals (day 14, C). ATPase histochemical staining (pH 10.2) was performed, which results in oxidative fibers stained light, in contrast to glycolytic fibers which stain dark. Representative images are shown; yellow arrows indicate ATPase cavities, and Serial sections were stained with hematoxylin and eosin (H&E). Scale bar represents 50 µm.
4.5 Discussion

Until this report, mechanisms of chronic muscle weakness in sepsis survivors could not be investigated due to lack of a translational animal model. Animal models of sepsis previously presented a major dilemma: considerably severe sepsis has to be induced in order to produce sustained muscle dysfunction, but such severity caused high mortality that largely prevented long-term experiments. Thus, currently existing animal models were either severe models (with insufficient or no resuscitation) causing early death of most animals, or were mild sepsis models which achieve high survival but not muscle weakness. Therefore the great majority of published studies on muscle weakness were completed within 72 hours of sepsis induction (Table 1.1; Holecek et al 2012) during which systemic inflammation and bacteremia were not resolved and thus cannot be considered “post-sepsis.”

Here, we show that animals rescued from otherwise lethal sepsis using our newly adapted ICU-like resuscitation protocol (Steele et al 2017) are 20% weaker compared to non-sepsis controls 2-weeks after sepsis induction (maximum specific force, \( p<0.001 \)), which to the best of our knowledge is the latest time-point investigated for analysis of tissue function. Evaluation of muscle strength in sepsis survivors is unique to our study where previously published reports either study muscle strength during sepsis, or conduct biochemical analyses on skeletal muscle after recovery from mild sepsis without conducting assessments on muscle function and therefore no associations could be made between molecular and functional data.

Further, we demonstrate that poor muscle quality, rather than loss of muscle mass, is responsible for the sepsis survivors' muscle weakness in the post-sepsis condition. Using body composition analysis, wet skeletal muscle tissue weight measurements, and cross-sectional area quantification of myofibers, we show that animals indeed have significant atrophy during sepsis, but that muscle mass recovers by the 2-week time-point (i.e. the time at which we observed muscle weakness). However to eliminate any possible discrepancy in muscle size among groups, force measurements were normalized to
physiological cross-sectional area (i.e. muscle size) to yield specific force measurements, which showed that indeed skeletal muscles from sepsis survivors have weakness even when adjusted for muscle quantity (Figure 4.3).

Additionally, our data suggests that polyneuropathy is not primarily responsible for muscle weakness in these murine sepsis survivors since we did not observe a rightward shift of the force-frequency curve in the low stimulation frequency range, which is a defining characteristic of polyneuropathy. Further, we did not observe evidence of denervation which typically results in fiber-type grouping (which would have been apparent in the ATPase histochemical stain and fiber-type labeling). Thus, we concluded that myopathy (i.e. dysfunction on the myofibrillar level) rather than atrophy or polyneuropathy is the major underlying cause of post-sepsis muscle weakness, which is consistent with clinical reports (Koch et al 2011).

Our pilot studies using this animal model of sepsis also indicate that activity is depressed in sepsis survivors for at least several weeks (Figure 4.7). Additional experiments using larger animal numbers and more sensitive and reliable methods of monitoring activity need to be conducted. We found that the infra-red motion detectors are highly variable when comparing the detectors to one another, thus making this a screening tool, rather than a robust method on which to base conclusions. Piezoelectric recording for sleep behavior and activity monitoring should be conducted in future experiments to follow-up on these preliminary findings.

Further, our data indicate that behavior-based assessments of strength, although desirable since euthanasia is not required, also lack sensitivity and repeatability and thus were discontinued in our studies. Other means of physical assessment, such as rotarod test for balance and coordination or treadmill running test for endurance, could potentially be used in future studies.

We believe that these findings are most relevant to the clinical situation due to careful use of our animal model. We adapted our newly established late-intervention ICU-like severe model of sepsis in which therapeutics (antibiotics and fluid resuscitation) are only initiated after the development of bacteremia,
and are continued twice daily for multiple days to treat the infection. Further, we used late middle-aged mice (16 months old), approximately equivalent to a 50 year old human (Flurkey et al 2007) which is more representative of the clinical population of sepsis patients and those who are highly vulnerable to post-sepsis dysfunction as well as the repercussions of such disability. Also importantly, we used the cecal slurry (CS) injection model to induce polymicrobial abdominal sepsis, as opposed to the surgical cecal ligation and puncture (CLP) model, mainly because mice that survive CLP-induced sepsis have a ligated cecum that may affect dietary habits and GI function, in addition to unresolved necrosis which may introduce variability in long-term physiological processes.

4.6 Conclusions

In summary, the present study demonstrates that sepsis triggers significant skeletal muscle weakness which persists long after recovery from sepsis pathogenesis itself (14 days after sepsis induction). Further, we show that muscle mass is recovered at this time-point, and no hallmarks of polyneuropathy were present, indicating that muscle quality, rather than quantity, is responsible for muscle weakness in the murine sepsis survivors. This conclusion was supported by abnormal muscle pathology as observed using ATPase histochemical stain. These data suggest that (A) the choice of animal model must be carefully made based on one’s scientific question, paying close attention to clinical translation, and (B) sepsis-induced skeletal muscle weakness is not due to muscle wasting, but rather dysfunction on the myofibrillar level.
CHAPTER 5

Mitochondrial Myopathy and Oxidative Damage are Associated with Chronic Muscle Weakness in Sepsis Survivors

5.1 Abstract

Published studies aimed at elucidating the underlying mechanism for muscle weakness in sepsis survivors have been limited to analysis during sepsis due to the lack of an appropriate model. Despite this limitation, these studies have consistently observed mitochondrial dysfunction during sepsis. The objective of the current study was to determine if mitochondria remain damaged and dysfunctional long after recovery from sepsis pathogenesis. Experimental sepsis was induced in 16-month-old mice by cecal slurry injection in combination with ICU-like interventions (antibiotic and fluid resuscitation). Two weeks later, animals were euthanized and skeletal muscles were processed for transmission electron microscopy which revealed enlarged mitochondria with gross morphological abnormalities in sepsis-surviving mice. Respiration analysis and evaluation of mitochondrial enzyme activities demonstrated that sepsis survivors have impaired mitochondrial function. As damaged mitochondria produce an abundance of free radicals, markers of protein oxidative damage (3-nitrotyrosine and protein carbonyls) were evaluated and found to be elevated in skeletal muscles of sepsis survivors. These novel findings indicate that long-term muscle weakness in sepsis survivors is accompanied by profound mitochondrial myopathy, which likely contributes to muscle weakness through reduced myofibrillar volume, decreased energy production, free radical production, and protein damage.
5.2 Introduction

The mechanisms of sepsis-induced muscle weakness have been poorly understood due to the lack of an appropriate animal model, which we addressed through refinement of the cecal slurry (CS) injection model of sepsis (Chapter 2) and ICU-like resuscitation protocol which results in high survival from otherwise completely lethal sepsis (Chapter 3). We then showed that sepsis surviving animals indeed have severe muscle weakness two weeks after recovery from sepsis pathogenesis (Chapter 4). Although loss of skeletal muscle mass (i.e. atrophy) during sepsis is a well-characterized pathophysiology and has been intensively studied in both humans and animals (Holecek 2012; Chai et al 2003; Jepson et al 1986; Choo et al 1989; Schefold et al 2010), we demonstrated that the murine sepsis survivors have muscle weakness even after atrophy is resolved (Section 4.4.2). Further, there are many documented clinical cases wherein long-term post-sepsis muscle weakness persists even without atrophy (Baldwin & Bersten et al 2014; Supinski & Callahan 2006; Eikermann et al 2006). Additionally, nutritional supplementation after sepsis often does not effectively cure skeletal muscle weakness (Lynch et al 2007; Lang et al 2007; Ash & Griffen 1989). These facts suggest that persistent post-sepsis weakness is likely caused by issues of muscle quality rather than quantity.

Many published studies on muscle dysfunction during sepsis in human patients and animal models demonstrated that sepsis induces mitochondrial dysfunction. Reductions in mitochondrial number (Zolfaghari et al 2015; Fredriksson et al 2006), electron transport chain enzyme activity (Fredriksson et al 2008; Callahan & Supinski 2005; Peruchi et al 2011), and glutathione (Cruzat et al 2014; Brealey et al 2004) have been reported, however it is unknown if such dysfunction remains unresolved after recovery from sepsis pathogenesis.

In the present study, we used our sepsis/ICU-like resuscitation protocol (Steele et al 2017; Chapter 3) in late middle-aged mice (16 months old, equivalent to 50 year old humans) as conducted in Chapter 4. Using this model, we show that skeletal muscle mitochondria are significantly enlarged and have frequent structural abnormalities. Further, we demonstrate that mitochondrial
function is significantly impaired long after sepsis is resolved, and that ATP content is reduced, demonstrating insufficient electron transport chain activity and energy production. Lastly, since damaged mitochondria produce an abundance of free radicals, we evaluate markers of oxidative damage and lipid peroxidation, demonstrating that skeletal muscle of sepsis survivors is characterized by significant oxidative damage. Together these data show that sepsis survivors suffer from mitochondrial myopathy and oxidative damage long after sepsis itself is resolved and are likely major contributors to long-term muscle weakness.

5.3 Experimental Approach

Late middle-aged mice (16 months old, male C57BL/6) were used for this study. Sepsis was induced by administration of a lethal dose of cecal slurry (CS) as described (Chapters 4 & 7) and the non-sepsis control animal received vehicle injection (10% glycerol-PBS) and resuscitation procedures. Animals were euthanized 14 days after CS or vehicle injection, and skeletal muscles were processed and analyzed using transmission electron microscopy (Section 7.13). Five electron micrographs were captured for the intermyofibrillar and subsarcolemmal mitochondrial populations, and mitochondrial area was quantified using ImageJ software. Mitochondrial DNA integrity was assessed using a classic PCR-based assay (Section 7.15) and quantified using densitometry analysis.

To evaluate mitochondrial function in skeletal muscle of sepsis survivors, two experiments were performed. First, non-sepsis controls and sepsis survivors were euthanized 14 days after CS injection and mitochondria were immediately isolated from the tibialis anterior (TA) and soleus muscles and subjected to respiration analysis using Seahorse (Section 7.14). Second, electron transport chain enzyme activity was assessed in sepsis survivors as well as in animals during sepsis, frozen muscle tissue sections processed for Figure 4.12 were used for a series of histochemical stains: nicotinamide adenine dinucleotide dehydrogenase (NADH; complex I), succinate dehydrogenase (SDH; complex II),
and cytochrome C oxidase (COX; complex IV). The result of these histochemical stains were quantified using Aperio ScanScope software.

Finally, sepsis-induced long-term oxidative damage was evaluated in protein isolated from the TA of non-sepsis controls and sepsis survivors. Nitro-oxidative damage was assessed by western blot for 3-nitrorytrosine, and protein carbonyls (i.e. lipid peroxidation) were evaluated using a commercial kit, and densitometry analysis was conducted (additional details provided in Section 7.17).

5.4 Results

5.4.1 Skeletal muscle mitochondria of sepsis survivors are characterized by gross structural abnormalities

We first used transmission electron microscopy (TEM) to observe structural and morphological integrity in the tibialis anterior of sepsis survivors (14 days after CS injection, ~10 days after bacterial clearance) and resuscitated-only controls (n=3 per group). This revealed gross morphological abnormalities among both the intermyofibrillar (Figure 5.1 A) and subsarcolemmal (Figure 5.1 B) mitochondrial populations in the sepsis survivors, including ruptured outer mitochondrial membranes, broken cristae, and vacuolar structures. The intermyofibrillar mitochondria (responsible for energy production for muscle contraction) were enlarged in the sepsis survivors, taking up 30.3 ± 2.7% more skeletal muscle area compared to controls (Figure 5.1 C) whereas this phenomenon was not observed in the subsarcolemmal population (Figure 5.1 D).
Figure 5.1 Sepsis surviving mice have increased mitochondrial volume density and disrupted mitochondrial integrity compared to non-sepsis controls. The extensor digitorum longus (EDL; oxidative hindlimb skeletal muscle) was harvested from murine sepsis survivors (2 weeks post-CS injection) and non-sepsis controls (n=3 per group) and processed for transmission electron microscopy. Representative micrographs of intermyofibrillar (A) and subsarcolemmal (B) mitochondria at 5,000X and 15,000X magnifications are shown (scale bars represent 500 nm). Mitochondrial area was quantified using ImageJ software, and was normalized to the non-sepsis controls (analysis of 5 images of each; C, D). Abnormal structure are indicated with the following symbols: broken cristae (stars), vacuolar structures (black arrows), disrupted outer membranes (white arrows), and fission/fusion (white arrowhead). Data expressed as means ± SEM.
5.4.2 The integrity of mitochondrial DNA is similar among sepsis survivors and non-sepsis controls

PCR analysis of mitochondrial DNA yielded two important conclusions: (1) mitochondrial copy number is not reduced in sepsis survivors compared to non-sepsis controls as indicated by no difference in short mitochondrial amplification among the two groups ($p=0.6$), and (2) long amplification of the “hot spot” region where mitochondrial DNA point mutations most often occur (Gatlier et al. 2006) was not different among sepsis survivors compared to controls ($p=0.5$). These results indicate that although sepsis survivors’ skeletal muscle mitochondria are damaged (Fig. 5.1), mitochondrial content seems unchanged and mitochondrial DNA is not damaged in the post-sepsis condition.

Figure 5.2. Mitochondrial DNA is not damaged in murine sepsis survivors’ skeletal muscle. DNA was isolated from the extensor digitorum longus (EDL) of sepsis survivors (day 14 after cecal slurry injection; n=11) and non-sepsis controls (n=10) and PCR was conducted to evaluate mitochondrial copy number represented by “short MT” as well as mitochondrial DNA damage represented by “long MT” (both adjusted to nuclear DNA). PCR products were visualized using ethidium bromide and quantified using densitometry analysis. Representative bands for non-sepsis controls and sepsis survivors are shown, and data is expressed as means ± SD. n.s. not significant (statistically).
5.4.3 Sepsis induces long-term impairment of mitochondrial function

Since our TEM analysis revealed mitochondrial damage in the skeletal muscle of sepsis survivors, and previous studies in patients and animals have revealed mitochondrial dysfunction during sepsis pathogenesis, we hypothesized that mitochondria fail to recover function even long after septic insult. We assessed mitochondrial bioenergetics using mitochondrial respiration on mitochondria isolated from the tibialis anterior of sepsis survivors (n=9) and non-sepsis controls (n=6) using the Seahorse Biosciences XF24 Flux Analyzer (method described further in Section 7.14; Zhang et al 2012). The sepsis survivors’ mitochondria were significantly impaired in respiratory capacity, where the ADP phosphorylation rate (State III) was only 72.7 ± 5.1% that of controls (p<0.01; Figure 5.3). Complex I- driven electron transport chain activity (maximal oxidative phosphorylation) was also significantly reduced in sepsis survivors (73.4 ± 6.5% of the rate of controls, p<0.05), with a similar trend observed for Complex II-driven activity (assessed by adding rotenone, a Complex I inhibitor). Additionally, State IV, in which ATP synthase is inhibited by addition of oligomycin, suggests increased electron leakage in the mitochondria from sepsis survivors. Together, these respiration results demonstrate that skeletal muscle mitochondria derived from sepsis survivors have significantly reduced respiratory capacity (~27% lower compared to controls), as well as electron leakage that may further exacerbate muscle dysfunction.
Mitochondria were isolated from the tibialis anterior of non-sepsis control (n=6) and post-sepsis (day 14, n=9) mice, and oxygen consumption rate (OCR) was measured in triplicate for each sample. Oxidative phosphorylation (state III) and complex I-driven electron transport (State V-CI) were significantly reduced in post-sepsis animals compared to controls, and a similar trend was observed in complex II-driven activity. State IV, in which the ATP-synthase inhibitor oligomycin is present, indicates there is greater electron leakage in the post-sepsis samples. Data is represented by means ± SEM. n.s. not significant (statistically).

Being that the respiration analysis was conducted using isolated mitochondria which selects for healthier mitochondria and therefore may blunt our understanding of the real effect of sepsis on mitochondrial health, a series of histochemical stains to evaluate mitochondrial enzyme activities were also performed on whole tissue sections (tibialis anterior; TA). This analysis was performed in non-sepsis controls and sepsis survivors, as well as animals during...
sepsis because we hypothesized that mitochondrial function may continue to
decline after sepsis due to the electron leakage which likely causes further
damage. Staining for complex I enzyme (NADH dehydrogenase) activity showed
weak, medium, and strongly stained fibers in the controls, due to the mixture of
fiber-types present in the TA (Figure 5.4 A). Nicotinamide adenine dinucleotide
dehydrogenase (NADH) activity decreased during sepsis (day 4; 62.0, 27.7, and
23.3% reduction in weak, medium, and strongly fibers, respectively; Figure 5.4
B, D), and further decreased in the medium and strongly active fibers in the
sepsis survivors (Figure 5.4 C, D). Similar step-wise reductions in enzyme
activity were observed in complex II (succinate dehydrogenase; SDH) activity
(Figure 5.4 E-H) and complex IV (cytochrome C oxidase) activity (Figure 5.4 I-
L). These data suggest that sepsis induces mitochondrial damage and
dysfunction (decreased respiratory capacity and reduced enzyme activity), and
that mitochondrial function is reduced even more after sepsis itself is resolved.
Figure 5.4 Mitochondrial enzyme activities are progressively reduced over time in sepsis survivors. Tibialis anterior specimens from experiments shown in Figure 4.11 were utilized for histochemical staining of mitochondrial enzyme activities. Staining for nicotinamide adenine dinucleotide dehydrogenase (NADH; complex I, A-C), succinate dehydrogenase (SDH; complex II, E-G), and cytochrome C oxidase (COX; complex IV, I-K) enzyme activity were performed. Quantification was conducted using Aperio ImageScope software (D, H, L), and values were normalized to the intensity of the controls for weak, moderate, and strong activity (exemplified as W, M, and S in panel A). Arrows indicate cavities of mitochondrial enzyme activity and point to the same fiber in serial sections. Data are expressed as means ± SEM. n.s. not significant. (statistically). Scale bars represent 50 µm.
5.4.4 ATP content is reduced in skeletal muscles of sepsis survivors

We next evaluated levels of the energy substrate adenosine triphosphate (ATP) in skeletal muscle of non-sepsis controls and post-sepsis mice. The preliminary results suggest that ATP tends to be reduced in sepsis survivors compared to controls in both the TA ($p<0.05$; Figure 5.5 A) and soleus (Figure 5.5 B). Three of the four post-sepsis samples which fell below the detection limit of the assay in the TA set were from the same animals which fell below the detection limit for the soleus set. This data, although preliminary, suggests that energy availability is decreased in the skeletal muscle of sepsis survivors which may be in part responsible for chronic muscle weakness.

Figure 5.5. Energy availability is reduced in the skeletal muscle of sepsis survivors. Vehicle or cecal slurry (CS) were injected to 16-month old mice, resuscitation procedures were followed for both groups, and animals were euthanized 14 days after injection. Snap-frozen skeletal muscles were pulverized, weighed, and deproteinated before conducting the bioluminescent assay for the ATP content in the tibialis anterior (A) and soleus (B) skeletal muscles, and Student's t-test was performed.
5.4.5 Oxidative damage is present in skeletal muscle of sepsis survivors weeks after initial insult

Sepsis pathogenesis stimulates oxidative damage due to accompanying hypotension and hypoxia, and dysregulated host inflammatory response to infection. Additionally, mitochondrial damage results in inability for mitochondria to buffer calcium and maintain their role in anti-oxidant activity; instead, damaged mitochondria release free radicals, which cause oxidative damage. Also, since the aged have reduced capacity to eliminate damaged proteins through proteolysis or autophagy pathways, we hypothesized that the sepsis survivors’ skeletal muscle would have persistent oxidative damage. Whole protein was extracted from the TA of control and post-sepsis animals, and western blot for 3-nitrotyrosine (3-NT) demonstrated that sepsis survivors have over 4X-higher nitro-oxidative damage than controls (Figure 5.6 A, \( p<0.001 \)). Further, oxidative damage was assessed by evaluating protein carbonyl (PC) formation using the OxyBlot kit, which similarly showed that sepsis survivors have >5X higher levels of PC moieties (Figure 5.6 B, \( p<0.01 \)) in both high and low molecular weight proteins. These results demonstrate that although heterogeneous, sepsis survivors have significant oxidative damage compared to controls, which has great implication for protein dysfunction.
Figure 5.6. Elevated oxidative damage is evident in skeletal muscle of sepsis survivors long after bacterial clearance. Protein was isolated from snap-frozen tibialis anterior muscles harvested from non-sepsis control animals and post-sepsis animals (14 days following cecal slurry injection). Western blot was performed for 3-nitotyrosine (3-NT; A) indicative of nitro-oxidative damage, and protein carbonyls were assessed using OxyBlot Protein Oxidation Detection Kit (B). Densitometry analysis was performed (C, D), and data is expressed as means ± SD.
5.5 Discussion

This study is the first which evaluated mitochondrial integrity and function in sepsis survivors rather than animals or patients during sepsis. As shown previously in Chapter 4, animals have profound muscle weakness long after recovery from sepsis pathogenesis. Further, muscle weakness persists even though muscle mass was recovered, demonstrating that chronic muscle weakness in sepsis survivors is primarily due to poor muscle quality rather than quantity. Here we show sepsis survivors suffer from mitochondrial myopathy and oxidative damage long after recovery from sepsis itself which are likely underlying mechanisms of post-sepsis chronic muscle weakness.

Such mitochondrial myopathy can contribute to muscle weakness in multiple ways. First, we found that intermyofibrillar mitochondria are enlarged and occupy 30% more muscle area compared to controls. This increase in mitochondrial volume area, likely an attempt to increase energy production from these damaged organelles, results in a decrease of myofibrillar content, which in itself contributes to muscle weakness.

Second, we show that sepsis survivors have decreased mitochondrial respiration by seahorse analysis and mitochondrial complex enzyme activities shown in whole tissue by histochemical staining. Further, adenosine triphosphate (ATP) content seems to be reduced in skeletal muscle of sepsis survivors, suggesting that sepsis survivors have reduced energy availability. As muscle contraction is an ATP-dependent process, decreased electron transport chain activity and reduced ATP content results in an energy crisis and reduced myofibrillar interaction.

In addition to these factors, mitochondrial myopathy contributes to muscle weakness through oxidative damage. Mitochondria produce an abundance of superoxide anions as a result of oxidative phosphorylation, and mitochondrial damage increases production of reactive oxygen species (ROS; Balaban et al 2005) which can cause further damage nearby proteins, lipids, and DNA (including to the mitochondria itself). We demonstrate that the skeletal muscle of sepsis survivors is indeed characterized by strong oxidative damage as indicated
by presence of 3-nitrotyrosine and lipid peroxidation (protein carbonyls). One limitation to the current study is that we have not identified the specific proteins that are damaged in the sepsis survivors’ skeletal muscle. However, the lower molecular weight proteins (<25 kDa) are likely mitochondrial proteins, whereas the higher molecular weight proteins are more likely to be sarcomeric proteins. Since oxidatively damaged proteins are commonly dysfunctional, such profound oxidative damage likely yields large consequences in both mitochondrial activity and sarcomeric interactions, again contributing to muscle weakness in the post-septic state.

The observed mitochondrial myopathy at this late time-point (2 weeks after sepsis induction) is that which remains after the natural system for mitochondrial quality control have done all that is likely possible. Although these processes are multifaceted and robust, they occur quite rapidly. Quality-control proteases eliminate small amounts of damaged proteins (Baker et al 2011), whereas mitochondrial fusion occurs when larger amounts of damaged molecules are accumulated. Fusion results in complementation so that defective processes for one of the original mitochondria are compensated by the other to restore ATP production. Fission in cultured fibroblasts resulted in complete redistribution of a mitochondrial green fluorescent protein (GFP) from one mitochondrion to the other within an hour (Youle et al 2012), suggesting that this process is extremely rapid. Debris and damaged molecules are often segregated, and fission takes place where the daughter mitochondrion which contains debris is hypopolarized and subject to the next level of quality control: autophagy (i.e. mitophagy). Interestingly, the state of starvation, which occurs during the acute phase of sepsis due to inactivity of the animals, can inhibit autophagy in attempt to increase ATP production (Rossignol et al 2004). Therefore, at this late time-point, endogenous pathways to restore mitochondria and their bioenergetics capacity have likely ensued, but may remain a potential therapeutic target to prevent or treat post-sepsis muscle weakness.
5.6 Conclusions

Significant mitochondrial damage and dysfunction result in reduced contractile machinery due to increased mitochondrial volume area, reduced mitochondrial respiration and energy production, as well as marked oxidative damage to skeletal muscle proteins, and these all together likely contribute to muscle weakness in sepsis survivors. Further work is necessary to verify that these phenomena persist to even later time-points, and to elucidate therapeutic strategies to prevent or repair such damage so that sepsis survivors are able to return to healthy and productive lifestyles.
6.1 Major findings

Chronic muscle weakness is a devastating phenomenon that threatens the quality of life and independence of the majority of septic shock survivors. Previously productive individuals are commonly discharged to nursing care facilities due to the development of functional limitations, including walking, cooking, bathing, or even going to bed. What's more, these individuals continue to acquire additional limitations at a higher rate after hospital discharge compared to individuals hospitalized for non-sepsis conditions.

Although clinicians and research scientists alike acknowledge this devastating trend, progress in the field has been limited due to the lack of a clinically relevant animal model of sepsis. The first aim of this project was set to resolve this critical barrier. First, we refined the cecal slurry preparation protocol to enable long-term storage without reduced bacterial bioactivity (Chapter 2). Next, we developed a late-intervention but aggressive ICU-like therapeutic resuscitation protocol which rescues the majority of young animals from otherwise lethal sepsis, yet still allows for the development of bacteremia, cytokinemia, and multi-organ injury (Chapter 3). We then adapted this sepsis/resuscitation protocol to middle-aged animals (16-months old, representative of a 50 year old human) as they are more representative of the clinical population, and found that sepsis surviving mice were ~20% weaker compared to non-sepsis controls two weeks after sepsis induction (Chapter 4). This muscle weakness was not attributable to atrophy as muscle size had recovered by this time-point indicating that post-sepsis muscle weakness was due to poor muscle quality rather than quantity (Chapter 4). A 30% increase in intermyofibrillar mitochondrial area was observed, as were striking structural defects and decreases in mitochondrial respiration and complex enzyme activities, indicating that sepsis survivors suffer from multifactorial mitochondrial myopathy (Chapter 5). Further, we showed that skeletal muscle of sepsis
survivors is characterized by marked oxidative damage as indicated by 3-nitrotyrosine and protein carbonyl levels, which likely have strong consequences on both mitochondrial and sarcomeric function.

Together these results highlight the importance of careful consideration of animal models in the research design for preclinical studies. More specifically, these results indicate that mitochondrial myopathy and accompanying oxidative damage are likely a primary underlying cause of chronic muscle weakness in sepsis survivors, whereby mitochondrial-specific anti-oxidant therapy may be a potential therapeutic strategy in recovery of muscle strength in sepsis survivors.

6.2 Future directions

6.2.1 Application of our newly refined protocols to specific and broad research questions

This model opens the door to answering broad research questions. First, it would be of interest to determine if the murine sepsis survivors have cognitive dysfunction as is reported in clinical patients (Iwashyna et al 2010). Along these lines, it would be interesting to evaluate changes in social behavior, as depression, anxiety, and post-traumatic stress disorder are more prevalent in sepsis survivors compared to the general population (Battle et al 2014). Additionally, alterations in circadian rhythms and sleep behavior would be another clinically relevant application of the model being that patients suffer from sleep disturbances which are first initiated during hospital stay during which abnormal secretion of melatonin is detectable (Mundigler et al 2002).

Second, since sepsis survivors have a high ongoing mortality rate following hospital discharge (Cuthbertson et al 2013) and since decreased physical function is linked to poor survival outcomes, this model could be used to evaluate timing and causes of mortality following acute recovery from sepsis, and their associations with physical decline. A potential downfall to this study is lack of a robust behavioral assessment of muscle strength (i.e. a method which does not require euthanasia). Pilot experiments using the treadmill endurance test or
rotarod challenge could be conducted as possible methods to evaluate physical function in sepsis surviving mice at multiple time-points until expiration.

Using this model, it could be possible to identify the causes of death in the non-survivors. To conduct this study, a large number of animals would be required since the mortality rate is ~30%. However, determining whether respiratory failure, which is the main cause of death clinically, is the common reason would further validate the clinical translation of our model and its application to preclinical experiments.

6.2.2 Further characterization of post-sepsis muscle weakness and cecal slurry

Now that this ICU-like sepsis/resuscitation model is developed, we can further characterize the long-term outcomes in sepsis survivors. In line with the current study, we plan to evaluate muscle strength in sepsis survivors at a later time-point. We will conduct a similar experiment to that in Figure 4.3 where groups of sepsis survivors and non-sepsis controls are euthanized and ex vivo analysis of strength is performed at 4 weeks and 8 weeks following sepsis. During these studies, we plan to utilize piezoelectric cage bottoms to assess not only activity, but also sleep behavior, throughout and after sepsis pathogenesis. One potential downfall is that mice would have to be individually housed, which may negatively impact general health as well as sepsis survival. These studies would confirm that sepsis survivors have muscle weakness that is chronic and resembles the clinical situation. These experiments would provide insight on changes in activity, sleep behavior, and circadian rhythms both during and after sepsis pathogenesis which would further characterize the post-sepsis condition using this novel animal model.

Next, we plan to confirm that the sepsis-induced muscle weakness phenotype observed in these studies using male C57BL/6 mice is not sex and/or strain dependent. We conducted a small pilot study using middle-aged female BALB/c mice which showed a similar trend in muscle weakness 2 weeks after sepsis (Figure 4.4), however it would be ideal to confirm the phenotype in female
mice of the same strain (C57BL/6) and also male mice of a different strain (i.e. BALB/c) separately.

To better understand the kinetics of muscle breakdown during sepsis and determine the phase at which muscle metabolism balance is restored, we will evaluate plasma markers of muscle catabolism throughout the sepsis time course, as we see atrophy during the acute phase but not the chronic phase (Figures 4.9-4.11). This simple experiment would help bridge the gap between the literatures on acute (i.e. during sepsis pathogenesis; ≤ 72 hours after infectious insult) and chronic studies (i.e. after bacteremia and systemic inflammation are resolved).

Our current work clearly demonstrates that skeletal muscle of sepsis survivors have profound oxidative damage (4-fold higher levels of 3-NT, and 5-fold higher levels of protein carbonyls; Figure 5.6), but we have two remaining research questions concerning oxidative stress in sepsis survivors. The first question concerns the timing of the oxidative damage to the skeletal muscle proteins. We hypothesized that oxidative damage to mitochondria first occurs during the acute phase of sepsis pathogenesis, and that the damaged mitochondria continue to generate ROS which causes further, and potentially accumulative, damage during the chronic phase. We are especially interested in answering this question, and plan on testing our hypothesis by using a transgenic mouse strain, MnSOD-TG, which overexpresses superoxide dismutase-2 (also referred to as SOD-2; manganese-dependent SOD, MnSOD; Yen et al 1996). Mitochondria of these mice are highly protected from oxidative stress, including that initiated by endotoxemia (Yen et al 1996; Choumar et al 2011). Our lab currently maintains a breeding colony of these mice, which was originally provided by Dr. Daret St. Clair, and we have confirmed significant overexpression of MnSOD in the skeletal muscle of these mice (Figure 6.1).
Figure 6.1. Skeletal muscle tissue of MnSOD-TG animals has nearly 3-fold higher expression of MnSOD. The gastrocnemius muscle was harvested and protein was isolated from untreated wild type and MnSOD-TG transgenic mice (n=2 per group). Western blot was performed for MnSOD and quantified using densitometry analysis. Data represent means ± SD.

Using these animals, we will determine whether mitochondria are the primary cause of sustained oxidative damage which will help identify promising therapeutic targets. Determining the time-points with the most severe oxidative damage (i.e. acute or chronic) will better establish the ideal time for therapeutic intervention.

The second major question regarding oxidative damage in the skeletal muscle of sepsis survivors that we hope to answer in the future concerns identification of the proteins which are damaged. Lower molecular weight proteins with 3-NT or protein carbonyl modifications observed by western blot (Figure 6.6) are likely mitochondrial proteins which are typically ≤ 25 kDa. These modifications would result in decreased mitochondrial function and therefore decreased energy production. On the other hand, modifications observed at higher molecular weights may be to sarcomeric proteins which could result in decreased force generation (Dalle-Donne et al 2003). Since we have candidate mitochondrial proteins, we will first conduct Western blot analyses with 3-NT and double stain the gel for mitochondrial complexes using fluorescently conjugated
secondary antibodies to determine if the complex proteins have nitrotyrosine residues. This would indicate that the complex(es) are nitro-oxidatively damaged and are likely dysfunctional. To identify other proteins which have oxidative damage, we will pull the proteins down by immunoprecipitation using 3-NT antibody, run by western, and use mass spectrometry to identify the proteins. This will give better understanding to the functional influence of oxidative damage to both mitochondria and sarcomeric function.

As our muscle function and molecular analysis studies were performed only in middle-aged animals, curiosity has developed as to how the results would differ in elderly animals or in young animals. We hypothesize that young animals may not develop chronic muscle weakness, whereas the elderly may be on the other end of the spectrum with increased severity and possibly the inability to recover muscle size, further contributing to development of muscle weakness. Therefore, I propose that a similar set of experiments to that conducted in Chapter 4 should be conducted in young (16-week-old, equivalent to 25 year old human) and elderly (22-24 month old, equivalent to 65 year old human; Flurkey et al. 2007) mice, whereby a lethal dose of CS is administered, the majority of animals are rescued. Muscle strength would then be assessed by ex vivo analysis and muscle size would be evaluated by body composition analysis, wet tissue weight, and cross-sectional area quantification. Results of such studies would not only yield better understanding of the effect of age on risk of long-term outcomes, but may contribute to more targeted/individualized therapeutic strategies.

It may be of interest to elucidate key differences in sepsis-induced muscle weakness and mitochondrial myopathy with a non-sepsis animal model of intensive care unit acquired weakness (ICUAW). Thus, comparisons could be made between our current data set with similar studies in disuse-mediated atrophy alone (mediated by hindlimb suspension) or non-sepsis infectious model, such as respiratory infection (mediated by inoculation of a drop of bacterial liquid culture in the mouse snare) or non-septic burn injury (mediated by scalding burn model). Understanding the extent to which atrophy and myopathy (specifically
mitochondrial myopathy) persist in these models of ICUAW would assist in elucidation of more targeted therapies for sepsis and non-sepsis mediated muscle dysfunction.

It would also be insightful to further characterize the cecal slurry which we make using our refined preparation protocol (Chapter 2). Although cecal slurry has been a common model of polymicrobial sepsis induction, it had been inconvenient due to the necessity of making CS fresh. We demonstrated that CS stored in glycerol-PBS at -80C maintains >99% bioactivity 6 months after storage, which we have found is true up to one year after storage (data not shown). We are currently planning an experiment to characterize the microbiome of the CS. We plan to conduct genomic studies to characterize the microbiota in both freshly made CS as well as frozen and thawed CS. Further, we plan to identify which bacteria circulate in the blood of animals with sepsis after CS-induced sepsis.

6.2.3 Further improvements to the model

Refinement of the cecal slurry preparation protocol which allows for long-term storage, increased reproducibility, and increased efficiency (Starr, Steele et al 2014), and the development of an ICU-like therapeutic resuscitation protocol (Steele et al 2017) now allows for development of organ dysfunction but still results in high survival so that long-term studies can be conducted. This model more closely mimics the clinical characteristics of patients, with lung and liver injury during the acute phase, as well as muscle weakness and sub-cellular damage during the chronic phase. However, this model can be further improved in several ways.

First, to control for the starvation period during the first 24-48 hours after sepsis induction, control animals could be pair fed. This would eliminate the influence of malnutrition on the development of muscle weakness; however, other groups have already shown that doing so does not result in atrophy and thus may be unnecessary (Voisin et al 1996).
Second, to mimic the period of bed-rest that patients encounter during stay in the ICU, hindlimb suspension could be included during the first ≤96 hours so that disuse-mediated mechanisms of muscle weakness may be triggered. Doing so is difficult, however, while performing resuscitation procedures every 12 hours as well as monitoring regularly for body weight and body temperature. An alternative may be to restrict movement by confining animals to a cage with significantly reduced area for movement. Although this does not achieve unloading of the skeletal muscles, the muscles would be exercised much less, making it more representative to a patient’s stay in the hospital.

Third, corticoid and/or neuromuscular blocks could be included as they are commonly used clinically. This would further complicate interpretation of results, however, being that these agents are suspected of contributing to muscle weakness. Therefore, we would suggest including these drugs only for specific research questions.

Fourth, inclusion of mechanical ventilation would further increase clinical translation of this animal model. Including mechanical ventilation would be especially important when studying diaphragm weakness during and after sepsis with concern for successful weaning from the ventilator. “Mouse ICU’s” have been established at some research institutions, however mechanical ventilation in mice remains a rare skill and thus would not likely be performed by the large majority of research groups who may otherwise use this sepsis/resuscitation model.

6.2.4 Use of transgenic animals to establish causal relationship between mitochondrial myopathy and weakness

We showed in Chapter 5 that sepsis survivors have mitochondrial myopathy and marked oxidative damage two weeks after sepsis induction, which we believe is the primary underlying cause of sepsis-induced chronic muscle weakness. A limitation of the current study is that this is associative in nature, not correlative or causative. Thus, to further establish the link between mitochondrial myopathy and its accompanying oxidative damage with muscle
weakness in sepsis survivors, we plan to utilize mice which are deficient in MnSOD (SOD-2; Van Remmen et al 1999; Yen et al 1996). MnSOD (SOD-2) is a mitochondria-specific superoxide dismutase which is an important anti-oxidant enzyme that protects the organelle from oxidative stress (Van Remmen et al 1999). MnSOD\(^{(-/-)}\) homozygous mutant mice are neonatally lethal (Li et al 1996); however heterozygous mutant MnSOD\(^{(+/-)}\) are born normally, have normal lifespans and breed well. We currently have a breeding colony of these MnSOD\(^{(+/-)}\) mutant mice, which were provided by Dr. Daret St. Clair, and we have confirmed that MnSOD\(^{(+/-)}\) mice have significantly reduced MnSOD expression in skeletal muscles (Figure 6.2).

![Figure 6.2. MnSOD deficient mice have reduced MnSOD expression in skeletal muscles.](image)

Figure 6.2. MnSOD deficient mice have reduced MnSOD expression in skeletal muscles. Similarly to the experiment shown in Figure 6.1 except using MnSOD knockdown transgenic mice, the gastrocnemius muscle was harvested, protein isolated, and western blot analysis performed to verify reduction of MnSOD expression in the skeletal muscle of these mice. Data represent means ± SD.
In a pilot experiment we found that young MnSOD\(^{(+/-)}\) mice had increased 3-NT generation in skeletal muscle compared to wild type mice upon induction of sepsis (data not shown) confirming increased sepsis-mediated oxidative stress in MnSOD\(^{(+/-)}\) mice. We will test our hypothesis that reduced anti-oxidant protection of mitochondria causes increased sepsis-mediated mitochondrial oxidative damage which results in more profound muscle weakness in MnSOD\(^{(+/-)}\) mice compared to that of wild type animals. With such results, we will conclude that increased mitochondrial damage via oxidative stress is linked to long-term muscle weakness in sepsis survivors.

6.2.5 Elucidation of therapeutics

This body of research indicates that use of anti-oxidant therapy may be most appropriate for preventing and/or ameliorating chronic muscle weakness after sepsis. We hypothesize that treatment of mice during or after sepsis with a mitochondrial-targeting anti-oxidant will prevent or reduce chronic muscle weakness. Bendavia (aka Elamipretide, SS-31) is a promising mitochondria-targeting anti-oxidant currently in clinical trials. Several mitochondria-targeting anti-oxidants have improved oxidative damage-related pathologies including cardiovascular and neurodegenerative diseases (Oyewole et al 2015). However, whether such anti-oxidants are effective at preventing and/or ameliorating chronic muscle weakness in sepsis survivors has not been determined.

Further, since not all oxidative damage is mediated by mitochondria, we hypothesize that additional treatment with a global anti-oxidant may further improve the efficacy. One such FDA-approved and readily available anti-oxidant is N-acetyl cysteine (NAC). First, it would be ideal to conduct proof-of-concept experiments by administering the anti-oxidant(s) immediately before and throughout sepsis pathogenesis, as well as during the post-sepsis phase to determine if muscle strength is maintained (as opposed to decreasing) and further validate that oxidative damage is largely prevented. The second phase of experiments would involve treatment with antioxidants during the resuscitation time-course to mimic receiving therapeutic intervention aimed at preventing loss
of muscle function while in the ICU; the third phase would involve delaying treatment until the chronic phase to determine if treatment can recover muscle function after weakness had already developed. One potential problem with including anti-oxidant treatment too early is that it may blunt important immune signaling and therefore alter severity and/or mortality.

Another potential therapeutic strategy involves activation of autophagy, which our preliminary data suggests may not be functioning adequately without pharmaceutical intervention. A low dose of rapamycin or resveratrol, which activates autophagy and mitophagy, may be used. We have evaluated LC3 (microtubule-associated proteins 1A/1B light chain 3B) which shows that autophagy is not appropriately activated in some survivors (Figure 6.3) supporting this alternative strategy.

![Image](image.png)

**Figure 6.3. Variable states of autophagy are present in skeletal muscle of sepsis survivors.** Control mice show LC3-I/II ratio greater than 1. Some post-sepsis mice show ratio less than 1 (post-sepsis lanes 3-5) indicating appropriate activation of autophagy, whereas others do not.
6.3 Overall summary

By developing this translational animal model of sepsis with delayed but aggressive ICU-like therapeutic intervention, we were able to evaluate muscle quantity and quality in sepsis survivors which previously was not possible. Using this new model, we were able to determine that murine sepsis survivors indeed have significant muscle weakness long after recovery from sepsis itself. We found that muscle weakness in the sepsis survivors is not attributable to loss of muscle mass, but instead is associated with mitochondrial myopathy and marked oxidative damage, clearly showing that muscle quality, rather than quantity, is responsible for long-term sepsis-induced muscle weakness. Our results indicate that anti-oxidant therapy is likely an effective therapeutic strategy to recover muscle strength after sepsis and allow patients to resume a high-quality and productive life.
CHAPTER 7
Thesis Methodology

7.1 Animals and husbandry

Male C57BL/6 mice were used for the large majority of these studies. Young adult (16-week-old) mice were acquired from The Jackson Laboratory for two purposes: (1) donors of cecal contents for cecal slurry preparation, and (2) experiments to establish the ICU-like resuscitation protocol after sepsis induction (Aim 1). Late middle-aged (16-month-old) mice were acquired from the National Institute on Aging for all other experiments. Animals were allowed to acclimate for at least 7 days prior to experiments to eliminate influence of transportation stress.

Animals were maintained in the Division of Laboratory Animal Resources at the University of Kentucky. Mice were housed up to 5 per cage in pressurized intraventilated (PIV) cages under controlled temperature (21-23°C), humidity (30-70%), and lighting (14/10 light/dark cycle) with free access to drinking water and chow (Teklad Global No. 2918, 18% Protein Rodent Diet, Madison WI). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. All animal handling techniques in these studies were performed as described and approved in our Animal Use Protocol #2009-0541 and were in accordance with the National Institutes of Health guidelines for ethical treatment.

7.2 Induction of chronic pancreatitis and diabetes in mice

To induce chronic pancreatitis in mice, recurrent acute pancreatitis was induced in young mice (6-months old) by a procedure modified from our previous protocol for acute pancreatitis (Okamura et al 2012). Each mouse received intraperitoneal (i.p.) injection with either physiological saline (control) or caerulein (American Peptide Company, Sunnyvale, CA) at a dose of 50 mg/kg body weight, 6-times hourly, 3 days per week (Monday, Wednesday, and Friday) for 9 weeks. Body weight of each mouse was monitored daily. To induce diabetes,
young (5-months old) fasted mice received streptozotocin (Sigma-Aldrich, St. Louis, MO) at a dose of 45 mg/kg body weight, i.p. once daily for 5 consecutive days. Control mice received no injection. Development of diabetes was confirmed by monitoring the non-fasted blood glucose level of each mouse by tail vein nick (ACCU-CHEK Nano blood glucose meter, Roche Diagnostics, Indianapolis, IN).

7.3 Cecal slurry stock preparation and characterization

Donor mice were euthanized by cervical dislocation and the ceca were immediately dissected (Figure 7.1 A), the cecal contents were removed (Figure 7.1 B) and slurry was made by suspending the cecal contents in 10% glycerol-PBS at a ratio of 1-mL for every 100mg wet cecal content weight. The slurry was passed through a series of sterile mesh strainers (Figure 7.1 C; 860, 380, 190, 74µm; Bellco Glass Inc., Vineland NJ) to remove debris. For maximal recovery, a sterile pestle was used to press the slurry through each strainer. Then, under continuous stirring using a magnetic stir bar, the slurry was dispensed into cryovials (Figure 7.1 D, 1-2mL each) and stored at -80°C in cell freezing containers which ensure slow (-1°C/minute) freezing to maintain bacterial viability. These procedures are summarized in a protocol flowchart (Figure 7.1 E) which we previously reported in Starr, Steele et al 2014 with minor modifications in Steele et al 2017.
Figure 7.1. Protocol for preparation of cecal slurry (CS) for cryopreservation. Cecal contents are aseptically collected from donor animals, suspended in 10% glycerol, and passed through a series of mesh strainers to remove debris. The slurry is aliquoted while stirring, and cryovials are slowly frozen using freezing containers at -80°C to preserve bacterial viability. Figure adapted from Starr et al 2014 and Steele et al 2017.
Cecal slurry was cultured on agar plates (containing 3.7% w/v brain-heart infusion broth and 1.5 % w/v agar) immediately upon stock preparation, and after storage at various temperatures (4, -20, and -80°C) for different periods of time (up to 6 months). The cecal slurry was diluted 100-times in sterile saline, plated on agar plates, and incubated for 24 ± 2 hours at 37 °C in ambient air. Colonies were counted and colony formation unit (CFU) per mL of cecal slurry was calculated.

In select experiments, bacteria were cultured on agar plates containing antibiotics which select for gram positive (azteonam) and gram negative (penicillin G) bacteria to further characterize the bacterial population in the cecal slurry. Further, cecal slurry was cultured on agar plates containing the antibiotic imipenem (IPM) at a physiological relevant concentration (relative to the concentration in the blood when injected to mice) to determine if the bacteria in cecal slurry were resistant to the antibiotic which was subsequently used for therapeutic intervention.

A CS-containing vial was thawed and heat-inactivated by incubating at 72°C for 10 minutes. The resulting heat-treated slurry was administered to mice (i.p.) to evaluate the severity of endotoxemia. A portion was also diluted per protocol, and cultured to confirm bacterial death. Such experiment was repeated in both young adult and late middle-aged animals.

7.4 Induction of polymicrobial abdominal sepsis by cecal slurry injection

Frozen cecal slurry (CS) stocks were thawed rapidly by agitating the cryovials in a 37°C water bath immediately before injection. The CS was mixed thoroughly and injected to mice intraperitoneally (i.p.) using a 25-gauge needle. To titrate the severity of the model, a series of different doses were administered to young, middle-aged, and aged mice, and survival was monitored. For all subsequent studies, a lethal dose of CS (determined to be 500µL in young adult mice and 400µL in late middle-aged mice) was administered to induce severe infection. In select experiments, vehicle-injected controls were administered 10% glycerol-PBS (i.p.). Survival was monitored daily for at least 10 days. Health
was assessed by body weight measurements and body temperature using a rectal temperature probe (P/N 4600-1.2.6 YSI) daily for 5 days and continued up to 14 days. Plasma blood glucose levels were also monitored in select experiments using the Accu-Check SmartView system.

7.5 Assessment of bacteremia

The skin over the tail vein was wiped with alcohol, nicked with a sterile razor blade, and 10µL of blood was collected, immediately diluted in 90µL of sterile saline, and spread onto agar plates. In selected experiments, half of the blood sample was spread onto agar plates containing antibiotics (2 mg/mL IPM), and the remaining half of the blood sample was spread on plates without antibiotics. Plates were incubated at 37°C for 24 ± 2 hours, colonies were counted, and CFU was calculated.

7.6 Therapeutic intervention

A significant portion of this dissertation project focused on the establishment of a novel ICU-like resuscitation model which resulted in rescuing the majority of animals from otherwise lethal cecal slurry-induced sepsis. Initial experiments included numerous methodologies: antibiotics, fluid resuscitation, pain management, temperature control, and attempted glucose control, which we initiated after bacteremia developed in all animals. Ultimately, a standard protocol was established and included administration of antibiotics and fluid resuscitation initiated 12-hours after CS-injection, and administered twice daily for 5 days thereafter. A summary of animals used for sepsis experiments shown in Chapters 3-5 can be found below in Table 7.1.

7.6.1 Antibiotics and fluid resuscitation

For antibiotic treatment, imipenem was used as it is a broad-spectrum antibiotic with potent capabilities for treating infection and is widely used in animal models of sepsis (Marques et al 2013; Kaynar et al 2014). Imipenem (IPM; stabilized in cilastatin) was reconstituted in sterile physiological saline for a
final concentration of 0.005mg/mL. The dose of IPM equivalent to the maximum
dose administered to patients in hospitals was determined to be 1.5 mg. IPM
was aliquoted and frozen at -20°C for up to 1 week (under these storage
conditions IPM maintained 95% efficacy). For each use, an aliquot was thawed
in a 37°C water bath and 1.5mg IPM (300µL) was administered i.p. to mice
beginning 1, 6, 12, or 24 hours after CS injection. The 1 and 6 hour groups
received another treatment 12 hours post-CS injection, and all groups then
received treatments every 12 hours for 5 days or until death.

In some experiments, fluid resuscitation (700µL, physiological saline, s.c.)
was administered alone or in addition to the 300µL of antibiotics beginning at 12
or 24 hours after CS injection. Antibiotics and fluids were administered twice
daily. Antibiotic therapy was continued for 5 days, and fluid resuscitation was
continued until body temperature recovered to at least 35.0°C.

7.6.2 Cage warming

In our initial experiments, temperature control was included as a
therapeutic treatment. Since rodents with sepsis become hypothermic, we
placed each cage on heating pads so that half of the cage was warmed mildly (≤
32°C) and the other half of the cage remained at room temperature, giving the
animals choice of environmental temperature. The cages were placed on the
heating pads at 12-hours, when the other resuscitation procedures were initiated.
<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Age</th>
<th>Terminal time-point (post-CS injection)</th>
<th>Group</th>
<th>Mice/group</th>
<th>Survivors</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial load, BT, survival</td>
<td>4 months</td>
<td>10 days</td>
<td>No antibiotics</td>
<td>6</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1h antibiotics</td>
<td>5</td>
<td>4</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6h antibiotics</td>
<td>5</td>
<td>3</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics</td>
<td>6</td>
<td>2</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24h antibiotics</td>
<td>5</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Bacterial load, BT, survival</td>
<td>4 months</td>
<td>10 days</td>
<td>No intervention</td>
<td>5</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics</td>
<td>7</td>
<td>2</td>
<td>29%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h fluids</td>
<td>8</td>
<td>1</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics &amp; fluids</td>
<td>9</td>
<td>7</td>
<td>78%</td>
</tr>
<tr>
<td>BT, survival</td>
<td>16 months</td>
<td>10 days</td>
<td>12h antibiotics &amp; fluids</td>
<td>9</td>
<td>6</td>
<td>64%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics, fluids, &amp; heat</td>
<td>9</td>
<td>4</td>
<td>44%</td>
</tr>
<tr>
<td>Bacterial load, BT, survival</td>
<td>4 months</td>
<td>10 days</td>
<td>No intervention</td>
<td>5</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24h antibiotics &amp; fluids</td>
<td>6</td>
<td>3</td>
<td>50%</td>
</tr>
<tr>
<td>Cytokines</td>
<td>4 months</td>
<td>24 hours</td>
<td>Non-sepsis control</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1h antibiotics</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6h antibiotics</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics &amp; fluids</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No intervention</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial load, BT, survival</td>
<td>16 months</td>
<td>14 days</td>
<td>No intervention</td>
<td>10</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12h antibiotics &amp; fluids</td>
<td>7</td>
<td>5</td>
<td>71%</td>
</tr>
<tr>
<td>Ex vivo muscle strength analysis</td>
<td>16 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resuscitated control</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-sepsis</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex vivo muscle strength analysis*</td>
<td>18 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-sepsis</td>
<td>5</td>
<td>2</td>
<td>40%</td>
</tr>
<tr>
<td>Behavioral muscle strength analyses</td>
<td>16 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sepsis</td>
<td>10</td>
<td>7</td>
<td>70%</td>
</tr>
<tr>
<td>Activity (wheel running)</td>
<td>16 months</td>
<td>7 days</td>
<td>Non-sepsis control</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sepsis</td>
<td>5</td>
<td>3</td>
<td>60%</td>
</tr>
</tbody>
</table>
Table 7.1 Animal cohort stratification by outcome measure with detailed sepsis survival rate, continued.

<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Age</th>
<th>Terminal time-point (post-CS injection)</th>
<th>Group</th>
<th>Mice/group</th>
<th>Survivors</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (infra-red motion detector)</td>
<td>16 months</td>
<td>3 weeks</td>
<td>Sepsis</td>
<td>20</td>
<td>14</td>
<td>70%</td>
</tr>
<tr>
<td>Body composition analysis</td>
<td>16 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>7</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measures of atrophy</td>
<td>16 months</td>
<td>4 days</td>
<td>Non-sepsis control</td>
<td>3</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seahorse analysis of MT respiration</td>
<td>16 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>6</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>16 months</td>
<td>14 days</td>
<td>Resuscitated control</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piezo activity &amp; ATP quantification</td>
<td>16 months</td>
<td>14 days</td>
<td>Non-sepsis control</td>
<td>7</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein expression</td>
<td>2-4 months</td>
<td></td>
<td>Wild type</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MnSOD-TgH</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wild type</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MnSOD KD</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Animals were separated by outcome measure, age, and time-point. Survival is given for sepsis groups. Filled columns indicate that survival rate is not applicable (i.e. non-sepsis groups or early terminal time-points). Animals which did not develop hypothermia below the cutoff (<30.0 °C) following cecal slurry injection were excluded from the study (n=11). BT: body temperature; MT: mitochondria. *Female BALB/c mice were used for this experiment.
7.7 Analysis of cytokinemia

Cytokinemia was evaluated in two sets of experiments: (1) to assess the influence of timing of therapeutic intervention on development of cytokinemia in young adult animals, and (2) to evaluate long-term low-grade inflammation in late middle-aged murine sepsis survivors. For the first experiment, blood samples were obtained from the IVC at the time of euthanasia (10% volume of 0.1M sodium citrate was used to prevent clotting during blood collection), and small blood samples (≤ 15 µL) were obtained during sepsis and in the sepsis-surviving animals by micropuncture of the tail vein for the second experiment. In both cases, blood was immediately centrifuged at 2,500 RPM at 4°C and plasma was stored at -80 °C until the biochemical assays were performed.

The proinflammatory cytokines interleukin-6 (IL-6), TNF-α, and IL-1β were evaluated, as well as the anti-inflammatory cytokine IL-10. IL-6 was quantified by ELISA, and the others were analyzed using a custom multiplex assay from Meso Scale Discovery, with the help of the University of Kentucky’s Center for Clinical and Translational Science (CCTS). This assay has increased sensitivity, allowing for quantitative measurement of even low concentrations, and also maximizes information gained from the same small sample. Samples were run in duplicate for the first experiment, which was not possible in the second experiment due to small sample size, but <2% variation in the duplicates for the standard curve encourages confidence in the assay.

7.8 Evaluation of lung and liver injury

In addition to evaluating cytokinemia to investigate the influence of timing of therapeutic intervention after CS injection on sepsis severity, lung and liver injury were also evaluated.

For histological analysis of lung injury, lungs were slowly infused with 10% buffered formalin phosphate from the trachea immediately after euthanasia as we previously described (Starr et al 2011; Okamura et al 2012). After fixation in 10% buffered formalin phosphate for 24 hours, the tissues were transferred to PBS and embedded in paraffin. Tissue sections (5µm thick) were cut, mounted on
glass slides, and stained with hematoxylin and eosin (H&E). Photomicrographs were taken, and histology was scored in a blinded semi-quantitative fashion adapted from the established method by Hirano et al. (2015) with slight modifications. Scoring was conducted for the degrees of (A) alveolar thickening, (B) cellular infiltration, (C) hemorrhage, (D) presence of debris, and (E) cellular hyperplasia with each assigned a score: 0 (normal; absence of markers), 1 (mild; sporadic pathological markers), 2 (moderate; frequent pathological markers) or 3 (most severe; ubiquitous pathological markers) which were totaled for a final score from 0 to 15.

For biochemical analysis of liver damage, plasma alanine aminotransferase (ALT) levels were quantified using a commercially available assay kit (Alanine Aminotransferase Activity Colorimetric/Fluorometric Assay Kit, Biovision). Samples were assayed in duplicate, and the duplicates were averaged to give one value per sample.

7.9 Assessments of muscle mass

To evaluate the kinetics of atrophy during sepsis and to monitor muscle mass during sepsis recovery, body composition analysis was performed using EchoMRI technology (Body Composition Analyzer, EchoMRI LLC, Houston, TX) before sepsis induction, and on days 2-5, 7, 10, and 14 after CS-injection. Utilization of this technology in this experiment also gave an understanding of changes in fat mass during and after sepsis.

The use of EchoMRI technology was especially useful in that changes in lean mass could be evaluated over time in the same animals; however, this technique is limited in that lean mass is measured in total, which skeletal muscle is only a part of, thus changes in skeletal muscle mass are not directly quantified. Therefore, we used the information gained using this body composition analysis to determine the time-point at which atrophy seems to be most severe (day 4). Then we performed an experiment in which a group of animals were sacrificed at this time-point (day 4) along with non-sepsis controls and 2-week post-sepsis animals. Hindlimb skeletal muscles of oxidative (soleus), glycolytic (tibialis
anterior, extensor digitorum longus), and mixed (gastrocnemius) metabolic phenotypes were dissected, blotted, and weighed.

7.10 Muscle strength analysis using an ex vivo system

To determine if our sepsis-surviving animals have muscle weakness long after sepsis was resolved, late middle-aged animals were injected with a lethal dose of cecal slurry and resuscitated beginning 12-hours later, and continued twice daily for 5 days. Sepsis surviving mice were euthanized 2-weeks later (14-15 days after CS injection; ~10 days after bacterial clearance), and muscle strength was assessed by force-frequency analysis on whole muscle samples. Non-sepsis controls and controls which received vehicle-injection and the resuscitation time-course were also included, and this experiment was repeated to ensure that the trends were reproducible.

At euthanasia, the right hind-limb was immediately placed in oxygenated Krebs-Ringer solution (details provided in Table 7.2; 95% O₂-5% CO₂, pH 7.4). The muscle bath was continuously oxygenated while the extensor digitorum longus (EDL) was dissected and tethers were placed on the proximal and distal tendons using braided silk suture (4-0). The muscle was freed from the leg, and mounted by attaching the tether at the distal end to a fixed hook and the proximal end to the lever arm of an ASI 300C-LR Aurora Scientific transducer system (Aurora, Ontario, CA, USA). The muscle was positioned between platinum electrodes and suspended in a temperature-controlled (25°C) chamber containing the Krebs-Ringers solution which was continually oxygenated, and allowed to acclimate for 5 minutes.
Using an Aurora stimulator (model 701C), the muscle was subjected to electrical field stimulation, and resulting force output was recorded using ASI 610A Dynamic Muscle Control software. By adjusting the EDL position, the maximum twitch force (at 1 Hz stimulation) was found, and digital calipers were used to measure the length of the EDL at this position which was defined as optimum length (Lo). Keeping the muscle at Lo, the force-frequency relationship was elucidated using stimulus frequencies of 1, 15, 30, 50, 80, 150, and 250 (250 Hz stimulations were most appropriate for maximum force production for the middle aged mice used in these studies, whereas 300 Hz stimulations are commonly used when using young animals). When the protocol was complete, the muscle was transferred from the apparatus to a muscle bath where the suture tethers were carefully removed from the muscle, then it was gently blotted dry and weighed. To calculate specific force, the physiological cross-sectional area was estimated according to Brooks & Faulkner (1988).
7.11 Behavioral analyses of strength and activity

In attempt to monitor changes in strength over time in the same animals, grip strength analysis (hindlimb and total) and inverted hanging test (Figure 7.2) were conducted before, during, and after sepsis induction by CS. Animals were subjected to three trials for each test, with at least a 10 minute rest time between hanging test trials, and the best (i.e. highest) trial was used.

Figure 7.2. A mouse performing the inverted hanging test as an assessment of muscle endurance and coordination. An animal is placed on a wire grid before it is inverted over top of 4 inches of bedding. Trial times are measured and recorded, with a maximum of 10 minutes at which time the trial is stopped. Animals which did not reach the maximum time rested for at least 10 minutes and were tested again. The best of 3 timed trials was used per animal.
Three pilot studies were conducted to assess activity in which animals were singly housed in (1) cages with running wheels where number of wheel revolutions were recorded, (2) cages with an infra-red motion detector on top which recorded the number of beam breaks (both wheel running and activity monitoring systems were interfaced with ClockLab data acquisition system acquired from Coulbourn Instruments), and (3) cages with Piezo equipment (Signals Solutions) which monitors respiratory rate.

7.12 Muscle histochemistry

7.12.1 Tissue processing

The tibialis anterior (TA) and soleus muscles were carefully dissected, embedded in a thin layer of optimal cutting temperature (i.e. OCT) compound, pinned at resting length to cork board, and immersed in liquid nitrogen-cooled isopentane. After the muscle was snap frozen, the cork board was transferred to a dry ice where the muscle was removed and quickly transferred to pre-chilled cryovials, and stored at -80°C. Serial tissue sections (8 µm) were cut, mounted, air dried, and stored at -20°C for short term or -80°C for long term storage.

7.12.2 Image acquisition

With the exception of immunofluorescently labeled sections, all histochemical staining results were observed using Nikon Eclipse E200 microscope and representative images were acquired using Nikon digital Sight DS-U3/DSFi1 digital software integrated with NIS Elements F3.2 Imaging Software (20X magnification).

7.12.3 Hematoxylin and Eosin (H&E) staining and quantification

For histological analysis of skeletal muscle samples, hematoxylin and eosin (H&E) staining was conducted. At least 100 fibers were observed and counted for the TA and all fibers were counted for the soleus, and fibers with at least one centralized nucleus were counted. The percent of fibers with centralized nuclei were calculated for each sample.
7.12.4 ATPase histochemical stain

For adenosine triphosphatase (ATPase) histochemical staining, frozen sections were fixed with pre-chilled acetone for 20 minutes at -20°C, subsequently thawed to room temperature, and placed in racks before being immersed in freshly-prepared pH 9.4 pre-incubation solution (0.02 Sodium Barbital, 0.036 M Calcium Chloride) for 15 minutes. The sections were rinsed in deionized water, and subsequently immersed in the adenosine triphosphate (ATP; 1.3 mM) incubation solution (6.7 mM sodium barbital, 6.0 mM calcium chloride) for 15 minutes. After washing in three changes of 1% calcium chloride (~3 minutes each), 10 minute incubation in 2% cobalt chloride, and five washes of ~5.0 mM sodium barbital, the sections were submerged in 2% ammonium sulfide for approximately 30 seconds. The sections were rinsed five times, dehydrated, and cover-slipped. It is important to note that the results of this assay are dependent on the pH of the pre-incubation solution; in these studies, the pH was adjusted to 9.4, which results in type 1 fibers stained light and type 2 fibers dark.

7.12.5 Immunofluorescent fiber-type staining and semi-automated quantification of cross-sectional area

For cross-sectional area analysis, fiber-types were determined by staining fiber-type specific isoforms of myosin heavy chain (MyHC) as previously described (Fry et al 2015). The frozen sections were thawed to room temperature and dried, rehydrated in PBS and subsequently incubated overnight at 4°C in the primary antibodies which were acquired from Developmental Studies Hybridoma Bank: MyHC type I (1:100; BA.D5; IgG2b), type Ila (supernatant; SC.71; IgG1), and type Iib (supernatant; BF.F3; IgM), and type IIX fibers remained unstained. Washing procedures were performed the following day (three washes in phosphate buffered saline, five minutes each), and sections were incubated in a cocktail of secondary antibodies comprised of 1:250 anti-mouse IgG2b conjugated with Alexa Fluor 647 (#A21242), 1:500 anti-mouse IgG1 conjugated with Alexa Fluor 488 (#A21121), and 1:250 anti-mouse IgM
conjugated with Alexa Fluor 555 (#A21426) for sixty minutes at room temperature. Sections were washed, post-fixed with methanol for five minutes, and mounted with vector shield mounting media (Vector, #H-1000). Entire muscle cross-sections were imaged using the tiles feature within Zeiss Zen 2 software (blue edition, v2.0, Zeiss, Oberkochen, Germany) interfaced with a Zeiss upright microscope (Zeiss, Oberkochen, Germany). Fibers were assigned as type I, IIa, and IIb depending on intensity within the Cy5, FITC, or Texas Red channels, respectively, and unstained fibers were identified as type IIx fibers. Cross-sectional area was analyzed on these whole cross-sections using an interactive semi-automated analysis program in ZEN (described above).

7.12.6 Histochemical staining of electron transport chain complex enzyme activities

To evaluate mitochondrial function in whole muscle tissue, a series of histochemical stains were performed which result in color deposit upon conversion of the substrate by the enzyme. The intensity of the staining depends on mitochondrial density as well as enzyme activity, and result in glycolytic fibers stained darker than oxidative fibers.

7.12.6.1 NADH dehydrogenase staining

To evaluate mitochondrial complex I activity, the standard NADH (nicotinamide adenine dinucleotide) protocol was followed in which thawed skeletal muscle sections were incubated in 2.4 mM NADH and nitro-blue tetrazolium in 0.5 M Tris buffer for 30 minutes at 37°C. After fixing using 10% phosphate buffered formalin, slides were placed in a rack and rinsed three times with deionized water, the sections were washed with a series of ascending then descending acetone solutions (30, 60, and 90% in deionized water). After rinsing three additional times in water, the sections were cover-slipped using aqueous mounting medium.
7.12.6.2 SDH staining

To evaluate mitochondrial complex II activity, the SDH (succinate dehydrogenase) histochemical staining protocol was followed in which sections were incubated in 100 mM sodium succinate salt and 1.2 mM NBT in 0.2 M phosphate buffer for 1 hour at 37 °C. The sections were fixed, rinsed, washed with acetone solutions, and cover-slipped as performed during the NADH staining procedure (detailed above).

7.12.6.3 COX staining

To evaluate mitochondrial complex IV activity, the COX (cytochrome c oxidase) histochemical staining protocol was performed in which sections were incubated with cytochrome C (1.1 mM), 342 mM sucrose, catalase (concentration), and DAB in 0.05 M phosphate buffer for 1 hour at 37 °C. Next, the sections were fixed, washed with deionized water, and dehydrated in ascending alcohols and two changes of safe clear, five minutes each while agitating, and were permanently mounted.

7.12.6.4 Image quantification using Aperio ScanScope software

Whole muscle cross-sections stained with NADH, SDH, and COX protocols were quantified using positive-pixel algorithms using ImageScope software which generates mean intensity values for weak positive, moderately positive, and strong positive fibers for each sample.

7.13 Transmission electron microscopy

The tibialis anterior were collected and multiple small pieces (1-2mm cubes) from each sample were cut and immersed in fixation buffer comprised of 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight. The tissue samples were post-fixed for 1 hour (1% osmiumtetroxide, 1.5% Potassiumferrocyanide), washed in triplicate (deionized water), stained for 1 hour (1% uranyl acetate), washed in duplicate, and dehydrated in ascending concentrations of alcohol (50, 70, 90, and 100% for ten
minutes each, with 100% alcohol step repeated twice) and 1 hour in propylene oxide. Samples were infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon, and samples were embedded in TAAB 812 (Epon) resin the following day and then polymerized at 60 °C for two days.

Using the Harvard Medical School Electron Microscopy Facility, ultrathin sections (approximately 60 nm) were cut (Reichert Ultracut-S microtome), picked up on copper grids stained with lead citrate (0.2%), and examined using a TecnaiG² Spirit BioTWIN transmission electron microscope. At least five fields of view of subsarcolemmal and intermyofibrillar mitochondria populations were captured for each sample using an AMT 2k CCD camera in a blinded manner.

ImageJ software was used to measure the area of the mitochondria in each micrograph. The total mitochondrial area per micrograph was calculated, and the ratio of mitochondrial to non-mitochondrial area was calculated to portray mitochondrial volume density in the skeletal muscle of each sample.

### 7.14 Mitochondrial isolation and respiration analysis

The right hind-limb was immediately placed in a muscle bath containing ice-cold isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin or BSA, 20 mM HEPES, 1mM EGTA; pH 7.2). The tibialis anterior (TA) was quickly dissected while the cold buffer was repeatedly pipetted on the muscle. Once freed, the muscle was transferred to a tube on ice containing the isolation buffer; all samples were collected in less than 2 hours. Trypsin was added (final concentration 0.25 mg/mL), the muscle was minced before homogenizing on ice in 3 rounds of 5 second intervals (total of 15 seconds; motor-driven Potter-Elvehjem homogenizer), and a protease inhibitor cocktail (Sigma #P8340; 15 µL) was added to the tissue homogenates. Mitochondrial pellets were obtained through two centrifugation steps at 4°C: the tissue homogenates were centrifuged for five minutes at 600 g, the resulting supernatant was decanted and then centrifuged at 5,000 g for ten minutes. The supernatant was discarded, and the pellet was suspended in isolation buffer, protein estimation was performed (BCA protein assay kit acquired from Thermo
It was previously shown that this method yields pure mitochondria being that the resulting fraction does not express transaminase (Patel et al 2009).

Mitochondrial function was assessed in the isolated mitochondrial populations through measurement of oxygen consumption rate (OCR) using Seahorse Bioscience XF24 extracellular flux analyzer, as previously reported (Patel et al 2014; Sauerbeck et al 2011; Zhang et al 2012) with minor modifications. The 24-well dual-analyzer sensor cartridges (Agilent Technologies, Santa Clara CA) were placed in a carbon dioxide-free incubator at 37 °C the day prior to the experiment. Once the mitochondria were isolated the day of the experiment, the Seahorse Flux Pak cartridges were filled in the following manner: (A) pyruvate, malate, and ADP (to yield final concentrations of 5 mM, 2.5 mM, and 1 mM, respectively), (B) oligomycin (1 µg/mL), (C) carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP; 3 µM), and (D) rotenone plus succinate (100 nM and 10 mM, respectively). The Seahorse instrument was calibrated as previously described (Patel et al 2014) using mitochondrial protein and respiration buffer (125 mM potassium chloride, 2 mM magnesium chloride, 2.5 mM potassium phosphate monobasic, 20 mM HEPES, and 0.1% BSA, adjusted to pH 7.2). The experimental plates contained both non-sepsis control and post-sepsis samples in triplicate, and were subjected to OCR analysis through the series of dispensing solutions containing substrates and inhibitors that were added to injector ports A-D. ADP phosphorylation (oxidative phosphorylation) was stimulated by the Nicotinamide adenine dinucleotide (NADH)-linked oxidative substrates pyruvate and malate and ADP (State III). State IV is entered after all ADP has been phosphorylated and ATP synthase is inhibited with the addition of oligomycin. The proton gradient is then collapsed by the addition of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and maximum electron transport activity is evaluated (Complex I-driven State V; NADH-driven respiration). Complex I is then inhibited with the addition of rotenone and Complex II-driven respiration is assessed by the addition of the substrate succinate (Complex II-
driven State V). Rates were generated using the AKOS oxygen consumption rate calibration algorithm; the average rate was determined for each sample.

### 7.15 Analysis of mitochondrial DNA integrity using PCR assay

To determine if sepsis survivors’ skeletal muscle had ongoing mitochondrial DNA damage, a PCR-based assay was conducted (Hunter et al 2010) with the assistance of Dr. Tadahide Izumi who graciously provided the necessary primers. DNA was isolated from snap-frozen tibialis anterior (TA) and extensor digitorum longus (EDL) samples from non-sepsis controls and 2-week post-sepsis groups using a spin-column kit from Invitrogen (PureLink Genomic DNA Mini Kit). The DNA concentration was estimated using a nanodrop spectrophotometer (ND-1000) which requires ≤ 2 µL of sample. Three sets of PCR reactions were performed: (1) short mitochondrial DNA, (2) long mitochondrial DNA which included the “hot spot” region where >80% of oxidative damage occurs, and (3) short genomic DNA (PCR conditions given in Table 7.2, primer design shown in Figure 7.3). After electrophoresis using a horizontal system (Life Technologies Horizon 11-14) using 1.5% agarose for short products, and 1.2% for long mitochondrial DNA, bands were visualized with ethidium bromide, and densitometry analysis was performed. To assess mitochondrial density, short mitochondrial DNA was normalized to short genomic DNA. To assess mitochondrial damage, long mitochondrial DNA was normalized to the short mitochondrial DNA.
Table 7.3 PCR conditions

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer sequence (5' → 3')</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear DNA</td>
<td>Forward: TATGGACCCCCCATGAGGAACA</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse: AACCGTCGGCTAAAGACGTG</td>
<td></td>
</tr>
<tr>
<td>Short Mitochondrial DNA</td>
<td>Forward: CCCAGCTACTACCATCATTCAAGT</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Reverse: GATGGTTTGGAGATTTGGTGTG</td>
<td></td>
</tr>
<tr>
<td>Long Mitochondrial DNA</td>
<td>Forward: AAAACTAGGCGCTCAGAGCATCCAC</td>
<td>6,918</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGAGTGATGATGTGAGGCATGTGCGA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7.3. Primer design for PCR-based assay of mitochondrial DNA damage.
7.16 ATP quantification

The tibialis anterior and soleus muscles from non-sepsis controls and 2-week post-sepsis animals were quickly dissected, placed in pre-chilled cryovials, and snap-frozen in liquid nitrogen before being stored at -80 °C. On the day of the assay, samples were pulverized in liquid nitrogen in a cryo-cup (BioSpec Products), the pulverized tissue was weighed in a pre-chilled cryovial, and lysis buffer was added (10 µL per 10 mg tissue) and samples were incubated at room temperature for 10 minutes. The samples were then deproteinized using perchloric acid (final concentration 1M PCA), neutralized with potassium hydroxide (pH 6.5-8 determined using litmus paper). The deproteinated samples were centrifuged at 2,500 RMP at 4°C, the supernatant was collected and kept on ice. The assay was performed directly after sample preparation was complete.

ATP content was evaluated using a luminescent commercially available kit using 50 µL of the supernatant, which was performed in duplicate for the TA, and in singlet for the soleus (due to low volume sample). ATP content was quantified using a standard curve.

7.17 Protein isolation and western blot analysis

Skeletal muscles were dissected, placed in cryovials, snap frozen in liquid nitrogen, and stored at -80°C until tissues were processed. Protein was extracted using the method described by Feng et al. 2012 with slight adaptations. Protein isolation buffer was comprised of 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, in 50 mM Tris base (pH 7.5), and a protease inhibitor cocktail was added (P2714, Sigma). The muscle samples were homogenized using mechanical homogenizers in approximately 20 volumes (w/v) of isolation buffer (250 µL and 1.5 mL for soleus and tibialis anterior muscles, respectively) at room temperature. The homogenates were then transferred to 1.5 mL boil-proof eppendorf tubes, and placed in an 80°C water bath for five minutes. The samples were then centrifuged at 12,000 g for 10 minutes at room temperature, the supernatant was transferred to a new tube and vortexed before aliquoting.
Due to the high detergent concentration of the isolation buffer, the protein concentration of the samples were evaluated using the Bio-Rad RC DC protein assay kit and a standard curve using IgG protein standard.

For evaluation of 3-nitrotyrosine residues, the control and post-sepsis skeletal muscle samples (20 µg loading protein) were resolved by SDS-PAGE electrophoresis (Invitrogen Xcell SureLock midi-system), and proteins were electrophoretically transferred (using iBlot system from Bio-Rad) to polyvinylidene difluoride (pvdf) membranes (pre-incubated in 100% methanol and allowed to dry for 5 minutes). The membranes were blocked for one hour in 5% milk at room temperature, and incubated in 1:3,000 3-nitrotyrosine primary antibody (Abcam #ab61392) overnight at 4°C. The following day, the membranes were washed, incubated in 1:10,000 anti-mouse antibody (Santa Cruz #2005) for one hour at room temperature, and washed again before chemiluminescent detection was performed (WesternBright ECL, Advansta Corporation). Densitometry analysis was performed on the resulting bands, and were normalized to Coomassie stain.

For evaluation of protein carbonyl formation in the samples, analysis was conducted using an OxyBlot kit (EMD Millipore, cat # S7150) using the given standard protocol with the minor adjustment of increasing the blocking time to a three hour incubation time. Densitometry and normalization procedures were as described above for 3-NT.

**7.18 Overall statistical analysis**

Survival curves were analyzed by Kaplan Meier LogRank test. Data for two-group comparisons were analyzed by Student’s t-test. When multiple comparisons were made, the Shapiro-Wilk normality test was run. If the data passed the normality test, one-way ANOVA and Holm-Sidak post-hoc test were used to analyze the data. Alternatively, when the data was not normally distributed, the Kruskal-Wallis test and Dunn’s post-hoc test were used. In instances where one group was assessed multiple times (i.e. bacteria load), repeated-measures one-way ANOVA was used, and in times when multiple
groups were assessed multiple times (i.e. body temperature data), repeated-measures two-way ANOVA was used and the Holm-Sidak post-hoc test was run. All data are expressed as means and standard deviations and p<0.05 was considered statistically significant. Statistical analyses were performed using SigmaPlot Statistical Software version 11.0 (Systat Software, San Jose, CA).
Appendix 1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NT</td>
<td>3-Nitrotyrosine</td>
</tr>
<tr>
<td>ADL</td>
<td>Activities of Daily Living</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CIM</td>
<td>Critical Illness Myopathy</td>
</tr>
<tr>
<td>CIP</td>
<td>Critical Illness Polyneuropathy</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal Ligation and Puncture</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CS</td>
<td>Cecal slurry</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonilcyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>FHL</td>
<td>Flexor halluces longus</td>
</tr>
<tr>
<td>GM</td>
<td>Gastrocnemius</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>ICUAW</td>
<td>Intensive care unit-acquired weakness</td>
</tr>
<tr>
<td>LD&lt;sub&gt;100&lt;/sub&gt;</td>
<td>100% lethal dose</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>i.p.</td>
<td>introperitoneal</td>
</tr>
<tr>
<td>IPM</td>
<td>Imipenem</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MOF</td>
<td>Multi-organ failure</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical research council</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>MT</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PC</td>
<td>Protein carbonyl</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential Organ Failure Assessment</td>
</tr>
<tr>
<td>SS-31</td>
<td>Szeto-Schiller-31</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
REFERENCES


VITA

Allison M. Steele

EDUCATION
University of Kentucky, Department of Physiology, Lexington, KY.
August 2012-present
Ph.D. expected December 2017

Franklin College, Franklin, IN.
August 2008-May 2012
Magna cum laude, GPA: 3.8 (4.0 scale)
B.A. in Biology, B.A. in Chemistry, Minor in Biochemistry

PUBLICATIONS
A. JOURNAL PUBLICATIONS


B. PUBLISHED ABSTRACTS


---

**SCIENTIFIC PRESENTATIONS**

**A. ORAL PRESENTATIONS**

1. **IAGG World Congress 2017**
   
   July 2017                    San Francisco, CA

   “Impairment of Mitochondrial Function in Murine Sepsis Survivors”

2. **40th Annual Conference on Shock**
   
   June 2017                    Ft. Lauderdale, FL

   “Mitochondrial Damage and Dysfunction in Skeletal Muscle of Middle-Aged Sepsis Survivors”

   *This abstract received a Presidential Travel Award.*
3. The Intercultural Honors-Undergraduate Scholar’s Day
May 2012 Franklin, IN
The Anti-Oxidant Cysteamine Confers a Cytoprotective Effect against Oxidative Damage in Saccharomyces Cerevisiae

4. The Intercultural honors-undergraduate Scholar’s Day
May 2011 Franklin, IN
“Investigation of the Antioxidant Cysteamine Using Yeast as a Model System”

B. POSTER PRESENTATIONS

1. 15th Biennial Advances in Skeletal Muscle Health and Disease Conference
March 2017 Gainesville, FL
“Mitochondrial Myopathy in Murine Sepsis Survivors with Long-Term Muscle Weakness”

2. The Gerontological Society of America’s 69th Annual Scientific Meeting
Nov 2016 New Orleans, LA
“Evaluating the Influence of Atrophy and Myopathy in Post-Sepsis Muscle Dysfunction”
This presentation received the George Sacher Student Award.

3. 39th Annual Conference on Shock
June 2016 Austin, TX
“Mice Rescued from Lethal Sepsis using an ICU-Like Resuscitation Protocol Exhibit Prolonged Muscle Dysfunction”
This abstract received a Travel Award.

4. 11th Annual CCTS Spring Conference
April 2016 Lexington, KY
“Myopathy-Dependent Long-Term Muscle Weakness in Murine Sepsis Survivors”

5. 38th Annual Conference on Shock
June 2015 Denver, CO
“Late Intervention with Repeated Antibiotic and Fluid Resuscitation Rescues Mice from Lethal Cecal Slurry-Induced Sepsis”
This abstract received a Travel Award.

6. 10th Annual CCTS Spring Conference
March 2015 Lexington, KY
“Middle-aged Mice Rescued from Lethal Sepsis by a New Late-Intervention Protocol Exhibit Long-Term Muscle Dysfunction”
7. **Department of Physiology Research Retreat**  
   July 2014                      Nancy, KY  
   “The Role of the Protein C Pathway in Protection of Pancreatic β-cells”

8. **Experimental Biology Meeting**  
   April 2012                     San Diego, CA  
   "The Anti-oxidant Cysteamine Confers a Protective Effect against Oxidative Damage in Saccharomyces cerevisiae in the Absence of Endogenous Repair Machinery"

9. **Indiana Academy of Science Annual Meeting**  
   March 2012                    Indianapolis, IN  
   “The Anti-Oxidant Cysteamine Confers a Protective Effect against Oxidative Damage in Saccharomyces cerevisiae”

10. **Wellesley College’s Summer Research Poster Session**  
    Aug 2011                   Wellesley, MA  
    “The Fabrication and Characterization of Gold Nanoparticle Thin Films”

11. **Butler University’s Undergraduate Research Conference**  
    April 2011                Indianapolis, IN  
    “The Anti-Oxidant Cysteamine Confers a Protective Effect against DNA Damage by the Oxidizing Agent Hydrogen Peroxide”

---

**RESEARCH SUPPORT**

1. **R01 GM126181** (Principal Investigator: Hiroshi Saito)  
   September 2017 – August 2021  
   Agency: NIH/NIGMS  
   “Chronic Muscle Weakness in Sepsis Survivors”

2. **F31 GM117868** (Principal Investigator: Allison M. Steele)  
   April 11, 2016-Current  
   Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship  
   Agency: NIH/GM  
   “Analysis of Chronic Muscle Weakness in Post-Septic Mice Using a Novel Resuscitation Protocol”

   Agency: The Franklin College Undergraduate Research Fund  
   “Determination of the Potential Protective Effects of the Anti-Oxidant Cysteamine in Saccharomyces cerevisiae”
HONORS and AWARDS

A. TRAVEL/SCIENTIFIC CONFERENCE AWARDS

1. University of Kentucky College of Medicine Student Travel Award
   July 2017
   Abstract title: “Impairment of Mitochondrial Function in Murine Sepsis Survivors”

2. Presidential Travel Award, 40th Annual Conference on Shock
   June 2017
   Abstract title: “Mitochondrial Damage and Dysfunction in Skeletal Muscle of Middle-Aged Murine Sepsis Survivors”

3. George Sacher Student Award
   Nov 2016
   Gerontological Society of America (Biological Sciences section)
   Abstract title: “Evaluating the Influence of Atrophy and Myopathy in Post-Sepsis Muscle Dysfunction”

4. University of Kentucky Graduate School Student Travel Award
   Nov 2016
   Abstract title: “Evaluating the Influence of Atrophy and Myopathy in Post-Sepsis Muscle Dysfunction”

5. Travel Award, 39th Annual Conference on Shock
   June 2016
   Abstract title: “Mice Rescued from Lethal Sepsis Using an ICU-resuscitation Protocol Exhibit Prolonged Muscle Dysfunction”

6. Travel Award, 38th Annual Conference on Shock
   June 2015
   Abstract title: “Late Intervention with Repeated Antibiotic and Fluid Resuscitation Rescues Mice from Lethal Cecal-Slurry Induced Sepsis”

B. SCHOLARSHIPS

1. Lilly Endowment Scholarship
   2008-2012
   Four year full-tuition scholarship inclusive of required course books. Awarded to two exemplary graduating high school seniors in each Indiana county.
2. Franklin College Trustee Scholarship

3. Holman Endowment Scholarship
   Awarded to exemplary Franklin College student majoring in Biology

4. Hoover Scholarship
   Awarded to exemplary Franklin College majoring in Chemistry

5. G & D Tucker Scholarship for Chemistry

6. Jordan Endowment
   Nominated by Dean of the College, Chair of Natural Sciences, and Head of the Chemistry Department. Recipient must commit to both academic excellence and leadership.

C. HONOR SOCIETIES

1. Alpha Lambda Delta
   Achievement of GPA ≥3.5 (4.0 scale) during first semester in college

2. Chi Beta Phi
   National interdisciplinary scientific honor society

3. Omicron Delta Kappa
   National leadership honor society

4. Order of Omega
   National honorary for Greek leaders (recognition of social sorority and/or fraternity leaders)

MEMBERSHIPS IN SCIENTIFIC SOCIETIES

The Gerontological Society of America (GSA), Student Member
Jan 2015-present

The Shock Society, Student Member
Jan 2015-present
**RESEARCH EXPERIENCE**

**Graduate Student:** Department of Physiology, University of Kentucky  
**2013-Present**  
Lexington, KY  
Mentor: Dr. Hiroshi Saito

During my training with Dr. Saito, the majority of my efforts have been aimed at: (1) Refinement of the cecal-slurry model of sepsis to allow long-term storage capability of the slurry, thus becoming a highly reproducible and time efficient model of polymicrobial abdominal sepsis; (2) Development of a late-intervention resuscitation protocol which can rescue the majority of animals from otherwise completely lethal sepsis; (3) Elucidation of skeletal muscle dysfunction in murine sepsis survivors long after sepsis itself is resolved.

**Research Experience for Undergraduates (REU), Chemistry Dept. of Wellesley College**  
**Summer 2011 Wellesley MA**  
Mentor: Dr. Nolan Flynn

The stability of gold nanoparticle thin films were determined using contact angle goiniometry. Experiments were aimed at determining the deposition orientation and substrate material which produced more stable thin-films.

**Student Researcher, Chemistry and Biology Departments of Franklin College**  
**Aug 2010-May 2012**  
Franklin, IN  
Mentors: Drs. Edward Chikwana and Sarah Mordan-McCombs

Using a yeast gene knockout library, the effect of the antioxidant cysteamine in its ability to protect living cells against oxidative damage in the absence of endogenous DNA repair machinery was investigated. The results of these experiments determined that cysteamine acts specifically within this glutathione-glutaredoxin pathway.

---

**TEACHING ACTIVITIES**

**A. COURSEWORK**

**PGY 615 SEMINAR IN TEACHING MEDICAL SCIENCE (MED SCIENCE TEACHING I).**  
A two (2) credit seminar course in which issues related to the theory and practice of life science education are discussed in a Socratic manner.
B. UNDERGRADUATE RESEARCH TRAINING

2016-current Stephanie Yamashita (currently Research Technician at the University of Kentucky)

2016 Damon Wallace (currently 2nd year medical student at the University of Kentucky)

2014-2016 Beverly Balasuriya (currently Research Technician at the University of Kentucky)

2014 Bill Hacker (Currently 4th year medical student at the University of Kentucky)

C. COURSES TAUGHT

Fall 2009 Franklin IN
New Student Leadership Seminar: LA 100 (1 credit hour), Franklin College

ACADEMIC SERVICE

New Student Planning Committee; Franklin College
May 2009-April 2010 Franklin, IN

This committee sought to improve an incoming student’s transition to the college through revising move-in day, new student orientation weekend, Franklin Gives Back community service day, and LA 100 (1 credit hour course which addressed the college’s mission, values, and value of a liberal arts education).

Senior Campaign Captain, Franklin College
Nov 2011-March 2012 Franklin, IN
Supervisor: Candice Moseley

Selected as a captain due to exceptional campus involvement; Sought “pledges to stay connected” from graduating seniors in which the students agreed to make small financial gifts to Franklin College after graduating.