Optimization of a Hibernation-Based Small-Volume Resuscitation Fluid

A DISSERTATION

SUBMITTED TO THE FACULTY OF

UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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September 2014

Acknowledgements

Firstly, I would like to give thanks where thanks are due, starting with my advisors, Dr. Matthew T. Andrews and Dr. Lester R. Drewes. They saw in me potential to be fulfilled when I was doing my rotations during my first semester at UMD and invited me to join the amazing life-saving project that is BHB/M.

A great deal of gratitude also goes to my Doctoral Committee members: Dr. Grant Anderson, Dr. Greg Beilman, and Dr. Ron Regal. All of our conversations, emails, and meetings have been invaluable towards my learning and growth.

None of the work presented here would have been possible without support from our funding agency, the U.S. Army Medical Research and Materiel Command (Grant W81XWH-11-1-0409).

I would also like to show appreciation to the people in my life who have showed me love and support through the years.

Those who are no longer with me: my role model since I was four years old, my brother Juan Isaac Castillo Flores; my grandfather, Eduardo Francisco Pérez de Lara y Garza Nieto; my mentor, José Nicolás de Tellitu Shultz, who always encouraged me to be better than him; and Prof. Sergio Ramiro Temblador Alcocer, who constantly pushed me to discover my intellectual limits, whether I wanted it or not.

My family: my parents Eduardo Francisco and Cecilia Dolores; my aunts Sandra, Ana, Adriana, and uncle Manuel; my cousins Manuel, Alejandra, Gerardo, Samantha, María José, and Martín. Above everyone else, thank you to my absolutely wonderful siblings, Eduardo Francisco, Sarah María, and Sofía Catalina for inspiring me to be a good role model.

My friends. I am lucky enough to say that I have friends in six continents, many of whom I am still in contact with, particularly those in Mexico, Chile, Australia, and my one very special friend in Ireland, who have managed through time and distance to always find the right words to keep me motivated. Thank you also to everyone at North Shore Tae Kwon Do for being my emotional outlet and helping me keep my stress level under control through the amazing activity that it is the practice of martial arts.

Last, but not least, I must thank my professors from the Universidad Autónoma de Nuevo León. Because of their teachings, I started this project already with a number of skills that proved valuable to my research such as knowledge in anatomy, histology, physiology, immunology, pathology, clinical pathology, surgery, statistics, and academic writing. Special thanks go to Dr. José Antonio Salinas Meléndez, whose advice has always led me to make decisions that have taken me to places I never thought I would be.

All in all, I just wish to thank everyone who has helped shape the person I am today. Even if your names are not listed here, you know who you are.

Dedication

Este trabajo está dedicado a mis hermanas, Sarah y Sofía.

Abstract

Background: Hemorrhagic shock is the number one cause of preventable deaths after trauma. Hibernation exemplifies a physiological state in which blood flow is reduced to a magnitude comparable to that of hemorrhagic shock. However, hibernators are naturally shielded from the insults that a similar reduction in blood flow would cause in a non-hibernating mammal. Our laboratory previously published a small volume resuscitation fluid based on hibernation physiology: BHB/M. It has three main components: 1) 4 M BHB, 2) 43 mM melatonin, and 3) 20% DMSO. Only the indicated concentration of each component of BHB/M has been previously tested. For that reason, worked towards the optimization of the composition and delivery of BHB/M in order to enhance survival in a rat model of hemorrhagic shock.

Methods and Results: Previously, BHB/M was given as a 1 ml/kg bolus followed by a 100 µl/hr slow infusion. BHB/M was administered as either a single bolus or a bolus plus slow infusion in acutely operated rats. There were no statistical differences (p>0.05) in mean survival times when comparing the bolus only (mean survival 496.67 ± 314.59 min. n=6) to the bolus plus slow infusion (mean survival 149.20 ±142.71 min. n=5) protocol.

Two separate dose-ranging studies were conducted for both BHB and Melatonin. In the BHB dose-ranging study, BHB was administered at either a 4 M,

2 M, or 0.4 M concentration in conjunction with 4.3 mM melatonin and 10% DMSO. 10-day mean survival showed a dose-dependent trend where the higher the concentration of BHB infused the longer the rats survived (4 M BHB, 7.38 ± 1.75 days; 2 M BHB, 5.25 ± 2.22 days; 0.4 M BHB, 2.07 ± 2.05 days). In the melatonin dose-ranging study, melatonin was administered at either a 4.3 mM, 0.43 mM 0.0043 mM, 0.000043 mM, or 0 mM concentration in conjunction with 4 M BHB and 2% DMSO. An osmolarity control composed of 4 M NaCl and 0.000043 mM melatonin in 2 % DMSO was also included. Administering 4 M BHB without melatonin resulted in very low mean survival times (4.38 ± 1.42 days); the same was true when infusing 0.000043 mM melatonin with 4 M NaCl (4.58 ± 1.42). All treatments containing both 4 M BHB and melatonin, regardless of concentration, resulted in mean survival times of ~7.5 days.

A large-volume experiment was conducted in order to compare isotonic BHB/M to the standard of care (LR). Rats subjected to 60% blood loss were infused with either LR, LR plus 4.3 mM melatonin, 140 mM BHB with $1.5x10^{-6}$ mM Mel, or 140 mM BHB with 4.3 mM Mel. LR and LR plus 4.3 mM melatonin had statistically higher ($p<0.05$) mean survival times (7.35 ± 1.59 days and 6.73 ± 1.57 days, respectively) than 140 mM BHB with $1.5x10^{-6}$ mM Mel (2.08 ± 1.18 days).

Conclusions: BHB/M can be administered as a single 1 ml/kg bolus; following the bolus with a 100 µl/hr slow infusion is not necessary to obtain a

maximum survival benefit. BHB must be administered at a 4 M concentration as there is a dose-dependent trend in which the lower the concentration of BHB administered the lower the percent survival to 10 days. Melatonin provides therapeutic effects at very low concentrations evident by the survival observed when administering a solution containing melatonin at a concentration a million-fold lower than previously published. Furthermore, melatonin is essential for survival since 4 M BHB without melatonin had a considerably reduced survival rate. A large volume dilute BHB/M is not a viable alternative to the standard of care.

Table of Contents

List of Tables xii

List of Figures xiv

CHAPTER I: INTRODUCTION 1

Biomedical Applications of Hibernation Strategies 3

Disuse Muscle Atrophy 4

Disuse Osteopenia 10

Insulin Resistance 15

Organ Preservation 20

Hemorrhagic Shock 23

Objective 31

CHAPTER II: MATERIALS AND METHODS 32

Animals 33

Anesthesia 33

Surgical Cannulation 34

Blood Volume Calculation 36

Induction of Hemorrhagic Shock 37
Optimization of Delivery 37
Optimization of Composition 40
Wound Closure 43
Experimental Treatments 43
Optimization of Composition 43
Monitoring of Physiological Constants 45
Whole-Blood Parameters 45
Histopathological Scoring 45
Plasma Parameters 47
Statistical Analyses 48
CHAPTER III: HYPERTONIC EXPERIMENTS 49
Survival 50
Optimization of Delivery 50
Optimization of Composition 51
Physiological Constants 59

Optimization of Composition 61
Whole-Blood Parameters 69
Optimization of Composition 69
Histopathological Scoring 88
Plasma Parameters 88
CHAPTER IV: ISOTONIC EXPERIMENTS 95
Survival 96
Three-Hour Shock 96
One-Hour Shock 98
Physiological Constants 101
Whole Blood Parameters 104
Histopathological Scoring 113
CHAPTER V: DISCUSSION 116
Future Directions 130
BIBLIOGRAPHY 135
APPENDICES 153

Appendix I: Blood Volume Experiments 154

Appendix II: Tissue Processing Protocol 157

Appendix III: Hematoxylin and Eosin Staining Protocol 158

Appendix IV: D-BHB Assay 160

Software Setup 160

Plate Setup 160

Procedure 161

Calculation 161

Appendix V: TNF-α Assay 163

Software Setup 163

Plate Setup 163

Procedure 164

List of Tables

Table 2.1. Isotonic resuscitation experimental design 44

Table 2.2. Histopathological scoring system. 47

Table 3.1. Melatonin Dose-Ranging Study I: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days. 52

Table 3.2. BHB Dose-Ranging Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days. 55

Table 3.3. Melatonin Dose-Ranging Study II: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days. 60

Table 3.4. BHB Dose-Ranging Study: Physiological constant data. 63

Table 3.5. Melatonin Dose-Ranging Study II: Physiological constant data. 67

Table 3.6. BHB Dose-Ranging Study: Whole-blood parameter data. 75

Table 3.7. Melatonin Dose-Ranging Study II: Whole-blood parameter data. 83

Table 3.8. BHB Dose-Ranging Study: Plasma BHB levels. 92

Table 3.9. Melatonin Dose-Ranging Study II: Plasma TNF-α levels. 93

Table 4.1. Three-Hour Shock Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days. 97

Table 4.2. One-Hour Shock Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days. 100

Table 4.3. One-Hour Shock Study: Physiological constant data. 102

Table 4.4. One-Hour Shock Study: Whole-blood data. 108

List of Figures

Figure 1.1 Regulation of protein synthesis and degradation in skeletal muscle. 8

Figure 1.2. Regulation of bone resorption and bone formation. 15

Figure 1.3. Insulin signaling. 20

Figure 2.1. Surgical Cannulation Procedure. 36

Figure 2.2 Optimization of delivery experiments: Experimental timeline. 39

Figure 2.3. Hypertonic resuscitation experiments: Experimental timeline..... 40

Figure 2.4. Isotonic resuscitation experiments: Experimental timeline..... 42

Figure 3.1. Optimization of delivery experiments: Kaplan-Meier plot of animals subjected to 60% blood loss..... 50

Figure 3.2. Melatonin Dose-Ranging Study I: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. 54

Figure 3.3. BHB Dose-Ranging Study: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. 56

Figure 3.4. BHB Dose-Ranging Study: Non-inferiority plot of animals subjected to 60% blood loss at 10 days..... 57

Figure 3.5. Melatonin Dose-Ranging Study II: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. 59

Figure 3.6. Melatonin Dose-Ranging Study II: Non-inferiority plot of animals subjected to 60% blood loss at 10 days. 61

Figure 3.7. BHB Dose-Ranging Study: Physiological constant data..... 62

Figure 3.8. Melatonin Dose-Ranging Study II: Physiological constant data..... 65

Figure 3.9. T1AM Pilot Study: Rectal temperature data. 69

Figure 3.10. BHB Dose-Ranging Study: Whole-blood parameter data..... 74

Figure 3.11. Melatonin Dose-Ranging Study II: Whole-blood parameter data.... 81

Figure 3.12. Melatonin Dose-Ranging Study II: Histopathological scores for (A) brain, (B) lung, and (C) small intestine..... 90

Figure 3.13. BHB Dose-Ranging Study: Plasma BHB levels. 91

Figure 3.14. Melatonin Dose-Ranging Study II: Plasma TNF-α levels..... 94

Figure 4.1. Three-Hour Shock: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days. 98

Figure 4.2. One-Hour Shock: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days..... 99

Figure 4.3 One-Hour Shock Study: Non-inferiority plot of animals subjected to 60% blood loss at 10 days..... 101

Figure 4.4. One-Hour Shock Study: Physiological constant data..... 103

Figure 4.5. One-Hour Shock Study: Whole-blood parameter data..... 106

Figure 4.6. One-Hour Shock Study: Histopathological scores for (A) brain, (B) lung, and (C) small intestine. 115

CHAPTER I: INTRODUCTION

Hibernation is an extended period of dormancy to avoid adverse winter (cold weather) conditions. Estivation is its summer (warm weather) counterpart. During hibernation and estivation, an animal is said to be torpid when dormant (Geiser and Mzilikazi 2011). Classic hibernation patterns include bouts of multiple days of reduced body temperature with scattered periods of transient normothermia. Normothermic events, known as interbout arousals (IBA), interrupt torpidity as the animals are briefly aroused from dormancy for periods of approximately 12 to 24 hours (Roots 2006); Geiser and Mzilikazi (2011). Hibernating animals pose an interesting model for human afflictions since during dormancy they encounter physiological challenges which would result in disease in a non-hibernating species, such as humans, without exhibiting adverse health repercussions (Roots 2006; Saey 2012). Though an animal cannot become torpid unless genetically programmed (Andrews 2007; Melvin and Andrews 2009), the pathways that regulate organ function are common to all mammals. It is through the modification of these pathways that hibernators overcome extreme physiological conditions (Carey, Martin et al. 2012).

There is a need to develop a new generation of biomedical technologies that take advantage of evolution and comparative physiology. Hibernation is an obvious mammalian adaptation that could support the development of new therapeutic regimes for the treatment of human ailments (Bouma, Verhaag et al. 2012; Carey, Martin et al. 2012; Saey 2012). In the last few years, the mechanisms underlying a

variety of pathways involved in the regulation of hibernation physiology, both during euthermia and torpor, have been elucidated, shining light into potential targets for therapeutic intervention. Furthermore, due to the extended periods of dormancy, the mechanisms by which the hibernating animal avoids morbidity and mortality are used chronically (long-term) without evident side effects, which cannot be said for the majority of drugs currently in the pharmaceutical market. However, only a handful of research teams are exploring the application of hibernation strategies for the resolution of human ailments. The focus of the research presented here is the development of new treatments and curative strategies based on hibernation physiology, particularly for hemorrhagic shock scenarios.

In this introductory section, first I will briefly illustrate some examples of diseases and conditions that hibernators manage to avoid. Then I will discuss hemorrhagic shock at length. Finally, the objective of the research presented in this document will be stated.

Biomedical Applications of Hibernation Strategies

Eighty-five years ago, the great comparative physiologist August Krogh (1929) wrote an eloquent paper in which he described disease as a functional issue to which physiological therapeutics must be developed. Dr. Krogh suggested that many of the numerous physiological questions we encounter could be answered by

studying the proper animal model, one that has evolved "adaptations of exquisite beauty and the most surprising character". He also acknowledged the need for an integrative approach to solving health issues through a collaboration between research scientists and clinicians. However, to this day the development of biomedical innovations, particularly those based on physiological adaptations of wild animals, are few. Furthermore, to our knowledge, there are no Food and Drug Administration (FDA) approved therapeutics for human use based on evolutionary adaptations such as hibernation despite the existence of a hibernating primate (Dausmann, Glos et al. 2004).

Disuse Muscle Atrophy

Physical inactivity can result in muscle atrophy. Disuse atrophy is an important clinical problem with no effective therapeutic regime (Glass 2003; Foletta, White et al. 2011; Bodine 2013). It can be due to a variety of reasons such as simple sedentary lifestyle, immobilization (Glass 2003; Jackman and Kandarian 2004; Lohuis, Harlow et al. 2007; Powers, Kavazis et al. 2007) (e.g. cast), suspension (Lohuis, Harlow et al. 2007; Lee, Park et al. 2008) (e.g. sling), bed rest (Lohuis, Harlow et al. 2007; Lee, Park et al. 2008), and even spaceflight (Lohuis, Harlow et al. 2007; Powers, Kavazis et al. 2007; Lee, Park et al. 2008).

Normally, muscle mass is maintained by a balance of protein synthesis with protein degradation. Disuse results in changes that decrease protein synthesis and

increase protein degradation, disrupting the balance required for the maintenance of muscle mass and hence leading to loss of myofibrillar content (Hudson and Franklin 2002; Lohuis, Harlow et al. 2007; Bodine 2013).

In non-hibernators, the discontinuance of muscle activity downregulates muscle-specific Insulin-like Growth Factor 1 (IGF-1) (Glass 2003; Cai, McCarron et al. 2004; Latres, Amini et al. 2005), inhibiting protein synthesis and enhancing protein degradation via downstream targets. IGF-1 is important in the activation of Phosphoinositide 3-Kinase (PI3K) and subsequently of Protein Kinase B (Akt) (Glass 2005; Kandarian and Jackman 2006; Vinciguerra, Musaro et al. 2010). Akt is involved in both the inhibition of protein synthesis and the enhancement of protein degradation. Inactive Akt promotes myofibrillar degradation by enhancing the activity of Fokrhead Box O (FOXO) proteins that induce the expression of Atrogin-1/Muscle Atrophy F-box (MAFbx) and Muscle Really Interesting New Gene Finger 1 (MuRF1) (Glass 2005; Kandarian and Jackman 2006; Vinciguerra, Musaro et al. 2010) which mediate ubiquination. MAFbx and MURF1 are considered master genes for muscle wasting and are regarded as the best markers for muscle atrophy (Foletta, White et al. 2011; Bodine 2013). Akt inhibits protein synthesis when it fails to phosphorylate Mammalian Target of Rapamycin (mTOR), a strong mediator of proliferative processes (Glass 2005; Latres, Amini et al. 2005; Kandarian and Jackman 2006). Furthermore, though the specific mechanisms have not been elucidated yet, immobilization increases oxidative stress. Reactive

Oxygen Species (ROS) accumulate as they are being produced faster than they can be eliminated (Powers, Kavazis et al. 2005), contributing to muscle atrophy via different pathways including 1) direct interaction with proteins and disruption of their structure and function (Hudson and Franklin 2002), thus making them more susceptible as substrates for catabolism; 2) interference with the activation of Akt, and hence all of its downstream targets, resulting in decreased protein synthesis (Vinciguerra, Musaro et al. 2010); and 3) direct inactivation of Inhibitor of Kappa B (IκB), allowing Nuclear Factor Kappa B (NF-κB) (Jackman and Kandarian 2004; Kandarian and Jackman 2006) to promote the expression of MAFbx and MURF1 (Glass 2005; Vinciguerra, Musaro et al. 2010) (Figure 1.1A).

Hibernators do undergo some loss of muscle mass during periods of inactivity. However, it is minimal compared to that experienced by non-hibernating organisms (Hudson and Franklin 2002; Lee, Park et al. 2008). Regulatory mechanisms exist in hibernators that give them the capacity to overcome muscle atrophy due to prolonged dormancy (Figure 1.1B and 1.1C). In most hibernating species, protein synthesis is decreased during periods of dormancy for two reasons: 1) translation cannot occur at the low body temperatures experienced during torpor (van Breukelen and Martin 2001) and 2) protein synthesis is costly in energetic terms (Bodine 2013). Consequently, most of the mechanisms by which hibernators maintain muscle mass must rely on the inhibition of proteolysis. This is supported by findings that gene expression and post-translational modifications of the proteins

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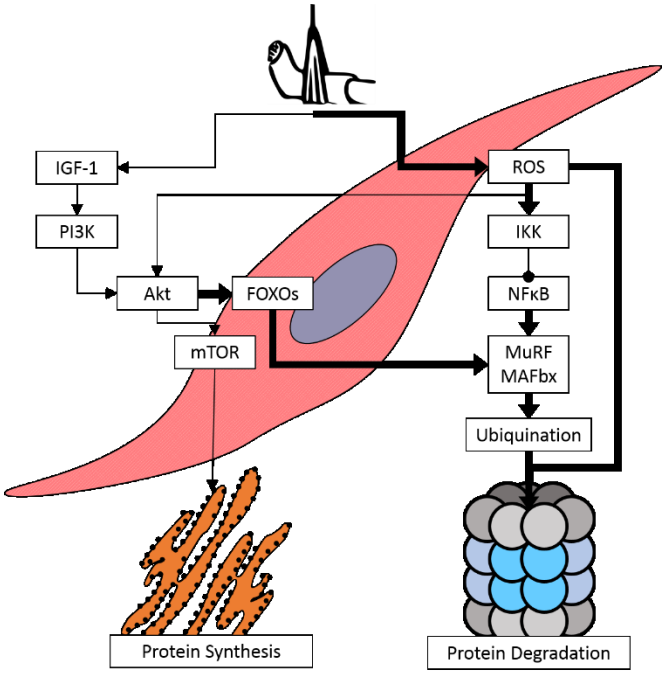
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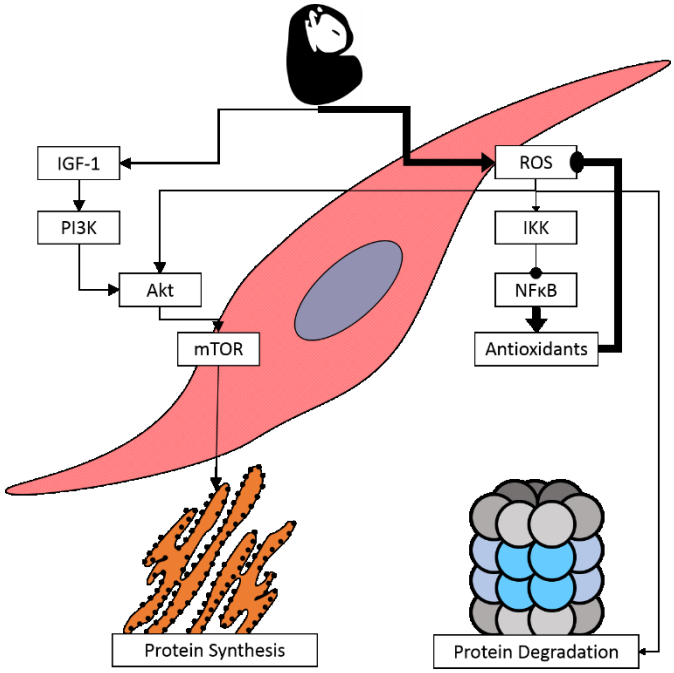
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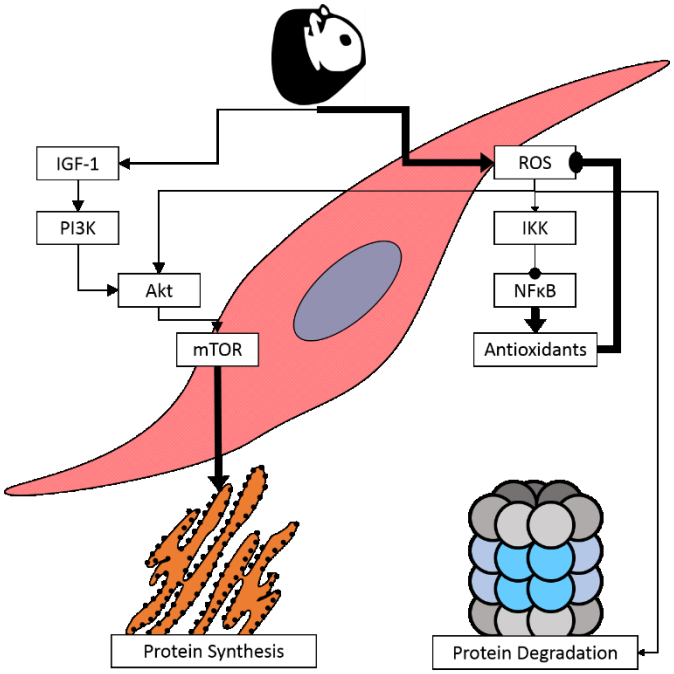

Figure 1.1 Regulation of protein synthesis and degradation in skeletal muscle.

In humans (A), muscle disuse reduces IGF-1 signaling which affects the PI3K/Akt pathway resulting in a reduction of protein synthesis. Furthermore, ROS are generated which enhance protein degradation directly and by pathways that promote ubiquination. Hibernating mammals in torpor (B) have the same patterns of reduced protein synthesis that humans exhibit during immobilization. However, their antioxidant activity is enhanced which tampers protein degradation due to oxidative damage. Furthermore, the master ubiquitin ligases of muscle atrophy, MuRF and MAFbx, are not present, impeding protein degradation via ubiquination pathways. During IBAs (C), mTOR activity is enhanced, promoting protein synthesis. Line width represents the strength of the interaction. Arrowheads represent promotion. Circle heads represent inhibition.

involved in the IGF-1/PI3K/Akt pathway are consistent with atrophy (Cai, McCarron et al. 2004; Bodine 2013). However, no changes in MAFbx and MURF1 have been observed (Rourke, Yokoyama et al. 2004; Lee, So et al. 2010; Barrows, Nelson et al. 2011). IBAs have been suggested as a means by which muscle protein

synthesis can occur and hence muscle mass can be spared (Lee, Park et al. 2008; Bodine 2013). This may be true since the mTOR anabolic pathway increases activity during arousal and decreases activity during dormancy (Lee, So et al. 2010; Wu and Storey 2012) (Figure 1.1C).

Markers of oxidative stress are upregulated during hibernation as is NF-κB (Carey, Frank et al. 2000). This might seem paradoxical when considering MAFbx and MURF1 are not present, or at basal levels, during dormancy. However, NF-κB is also involved in the transcription of endogenous antioxidants such as Manganese Superoxide Dismutase (MnSOD) (Tanaka, Matsumura et al. 2002; Briganti and Picardo 2003), suggesting NF-κB might confer protection against exacerbated oxidative damage, hence modulating oxidative stress.

Finding a treatment for muscular atrophy is the focus of a number of research groups (Jackman and Kandarian 2004; Glass 2005; Kandarian and Jackman 2006). Some of them use an IGF-1/PI3K/Akt approach (Kaspar, Lladó et al. 2003; Stitt, Drujan et al. 2004). For example, they administer IGF-1, maintaining activity of all of its downstream targets. Others focus on the modulation of oxidative stress as a therapeutic target, particularly on the effect of Vitamin E (Appell, Duarte et al. 1997; Servais, Letexier et al. 2007) and other antioxidants (McClung, Kavazis et al. 2007).

Though many researchers have suggested the investigation and application of hibernation strategies to avoid or minimize muscular atrophy (Harlow, Lohuis et al. 2001; Hudson and Franklin 2002; Fuster, Busquets et al. 2007), no biotranslational studies are currently engaged to our knowledge.

Disuse Osteopenia

Bone tissue is in constant turnover, renewing and repairing continuously through remodeling. Bone mass is maintained through balanced osteoblastic and osteoclastic activities (Komarova, Smith et al. 2003).

Similarly to muscular disuse atrophy, lack of physical activity may result in osteopenia or disuse osteoporosis (Jenkins and Cochran 1969). The absence of pressure forces to the bone results in an imbalance in bone remodeling where bone resorption exceeding bone formation (Jenkins and Cochran 1969; Sakai 1999; Donahue, McGee et al. 2006; McGee-Lawrence, Carey et al. 2008; Alexandre and Vico 2011). Despite differences in the mechanisms of bone degradation between disuse and postmenopausal osteoporosis (Donahue, Vaughan et al. 2003), the current treatment for patients with osteopenia, regardless of cause, are the same (Sakai 1999).

The pathophysiology of immobilization-induced osteopenia is not thoroughly elucidated yet. We know that it involves both mechanical and endocrine/neuroendocrine cascades. Unloading signals the inhibition of Nitrous

Oxide (NO) production (Jenkins and Cochran 1969). Low levels of NO upregulate osteoblastic expression of Receptor Activator of NF-κB Ligand (RANKL) which promotes osteoclastogenesis and bone resorption (Donahue, McGee et al. 2006; Rubin, Rubin et al. 2006; Lau and Guo 2011). Leptin has both osteogenic and anti-osteogenic functions, depending on where its receptor is expressed (Alexandre and Vico 2011; Doherty, Florant et al. 2014). Its osteogenic function involves signaling the hypothalamus to stimulate the production of Cocaine and Amphetamine Regulated Transcript (CART) protein which downregulates the expression of RANKL, inhibiting osteoclast differentiation and bone resorption. The anti-osteogenic effects of leptin upregulate RANKL, promoting osteoclast differentiation and bone resorption. Insulin also plays a role in bone resorption. It can directly stimulate leptin release from adipocytes and decrease osteoblastic Osteoprotegerin (OPG) expression (Jenkins and Cochran 1969; Cawley, Yanik et al. 2010; Doherty, Florant et al. 2014). Since OPG is a decoy receptor of RANK (Vega, Maalouf et al. 2007), its downregulation allows increased RANK/RANKL binding (Lau and Guo 2011). All the resorptive processes create an acidic milieu in the bone that removes a carboxyl group from osteocalcin, creating a positive feedback loop with osteoclacin as it increases insulin production in the β-cells of the pancreas (Cawley, Yanik et al. 2010; Doherty, Florant et al. 2014) (Figure 1.2B).

Some bone loss does occur in certain hibernating mammals (e.g. ground squirrels, golden hamsters, and little brown bats) (Donahue, Vaughan et al. 2003; Donahue, Galley et al. 2006; Donahue, McGee et al. 2006). However, at least two species of bears preserve bone mass and structure despite long periods of immobilization by maintaining a balance in the remodeling processes (Donahue, Vaughan et al. 2003; Donahue, McGee et al. 2006; McGee-Lawrence, Carey et al. 2008; McGee-Lawrence, Wojda et al. 2009; Alexandre and Vico 2011). Identifying how hibernators overcome bone loss during unloading could lead to the development therapeutics for osteopenia.

Because there is a difference in hormone patterns between hibernating species, it is difficult to draw generalized conclusions for all hibernators. For that reason, and since bears seem impervious to disuse-induced osteopenia, we will describe some of the mechanisms involved in the maintenance of bone tissue in the hibernating bear (Figure 1.2C).

The skeleton of bears somehow perceives that it is loaded during hibernation (Seger, Cross et al. 2011), maintaining a constant production of NO (Doherty, Florant et al. 2014). Osteogenic leptin is increased in early hibernation (Donahue, McGee et al. 2006; Doherty, Florant et al. 2014), upregulating CART expression and maintaining RANKL expression at low levels (Doherty, Florant et al. 2014). Insulin levels are low during hibernation (Bradford 2010; Doherty,

Florant et al. 2014), impeding insulin-induced downregulation of OPG expression. These mechanism work in coordination to maintain a balance between osteoblastic and osteoclastic activities and hence preserve bone mass.

The mechanisms of immobilization-induced osteopenia also appear to involve Parathyroid Hormone (PTH), a hormone that developed along with the evolutionary process that allowed animal development from aquatic to terrestrial life (Sakai 1999). Researchers identified 9 amino acid residue differences between human PTH (hPTH) and black bear PTH (bbPTH) (Donahue 2009). They have successfully cloned and recombinantly produced bbPTH and used it to prevent bone loss in a mouse model of Duchenne muscular dystrophy. bbPTH has greater osteoanabolic effects than hPTH (Gray, McGee-Lawrence et al. 2012).

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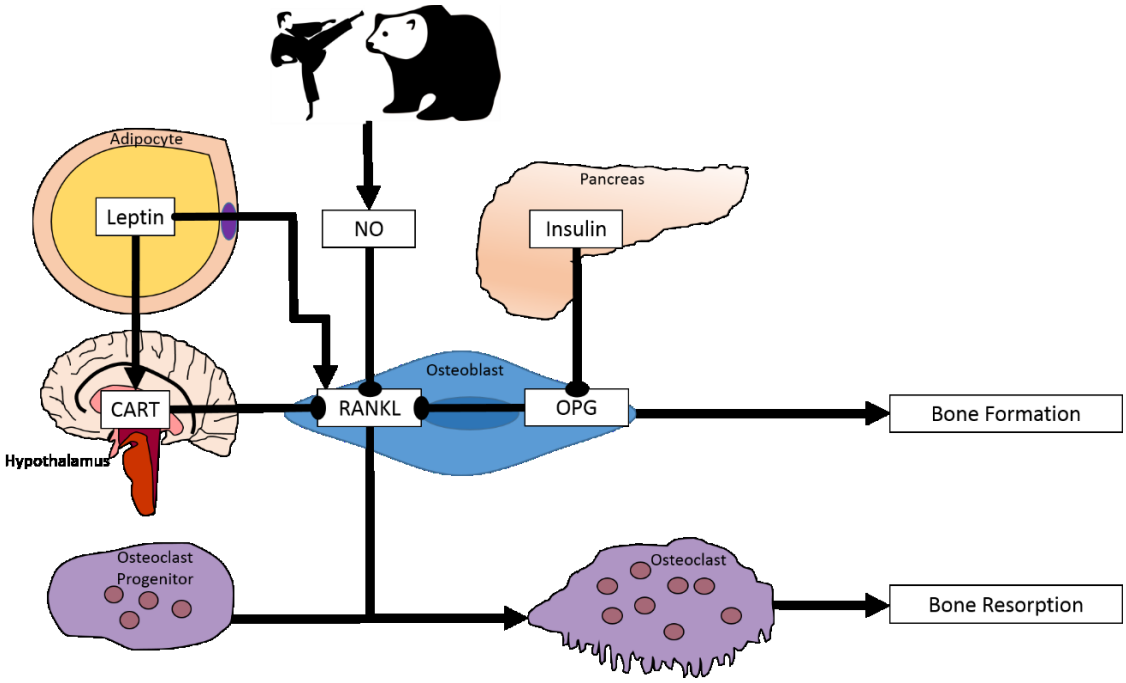
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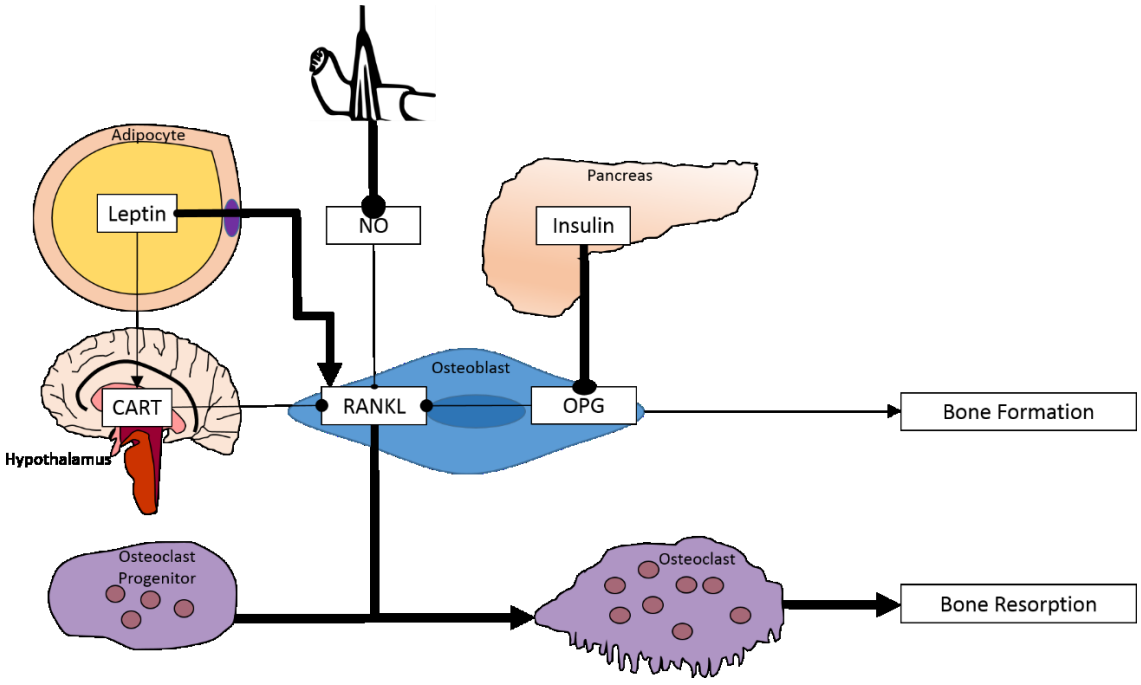
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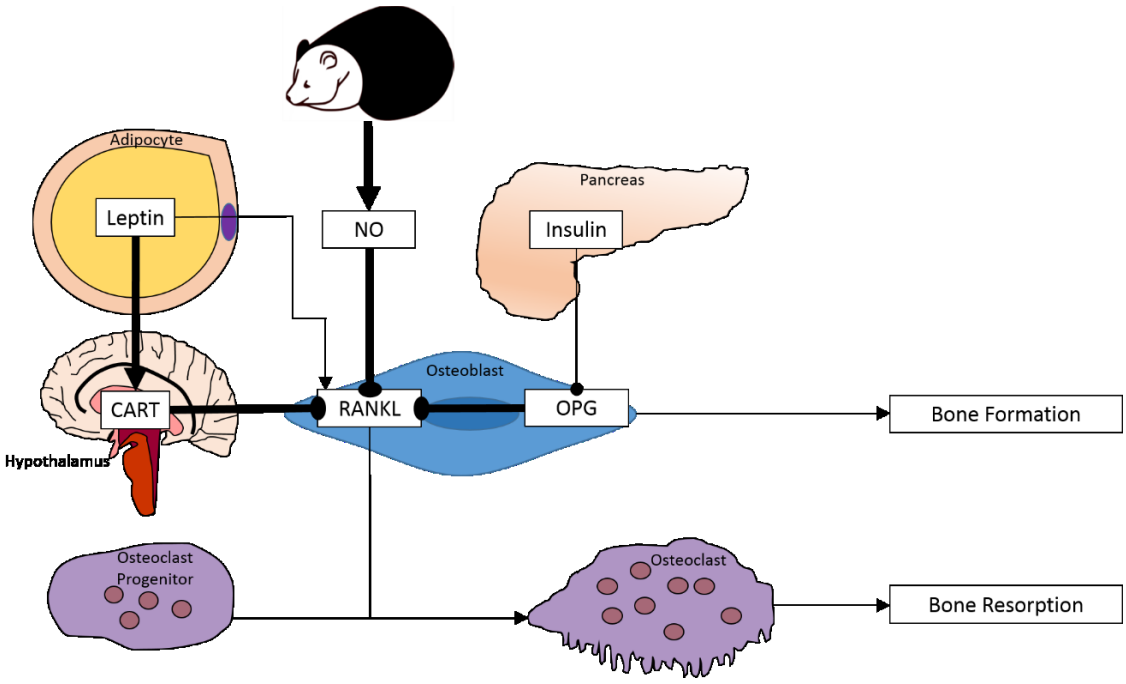


Figure 1.2. Regulation of bone resorption and bone formation.

During normal mechanical loading (A), RANKL receives both promoting and inhibitory signals, maintaining bone formation and bone resporption in balance. Immobilization in humans (B) enhances the promoting signals of RANKL and reduces its inhibitory signals, promoting an imbalance favoring bone resorption which results in osteopenia (B). Hibernating bears (C), maintain a balance between bone resoprtion and bone formation. However, bone turnover rates are decreased. Line width represents the strength of the interaction. Arrowheads represent promotion. Circle heads represent inhibition.

Insulin Resistance

Fat-storing hibernators increase their body mass to volumes that would lead to serious metabolic alterations, such as insulin resistance (Bloomgarden 2007; Saey 2012). Ample evidence suggest a relationship between obesity-induced

insulin resistance and the development of type 2 diabetes (Kadowaki 2000; Shulman 2000; Marette 2003; Puigserver and Rodgers 2006; Bloomgarden 2007).

In a normal physiological state, eating increases in plasma glucose which stimulates insulin secretion, promoting glucose metabolism and storage (Puigserver and Rodgers 2006) (Figure 1.3A). The interaction of insulin with its receptor activates Insulin Receptor Substrates (IRS) which in turn promotes PI3K/Akt pathway activation (Shulman 2000; Marette 2003; Puigserver and Rodgers 2006). Akt is important in the regulation of plasma glucose levels as it 1) promotes the translocation of Glucose Transporter 4 (GLUT4), the insulin regulated glucose transporter, to the cell membrane (Shulman 2000; Marette 2003) allowing circulating glucose to enter the cells where it can be stored as glycogen in striated muscle or as triglycerides in adipose tissue; 2) inhibits mTOR, which downregulates IRSs (Bloomgarden 2007); and 3) phosphorylates FOXOs which stimulate β-oxidation and gluconeogenesis (Kadowaki 2000).

Insulin resistance is a pathology characterized by an unsuccessful response of target tissues to insulin (Puigserver and Rodgers 2006; Bloomgarden 2007) (Figure 1.3B). Insulin production is normal, but the cells cannot use it efficiently, hence glucose is not metabolized leading to hyperglycemia (Inzucchi 2002; Marette 2003). β cells in the pancreas respond by increasing insulin production to compensate, contributing to the development of hyperinsulinemia (Bloomgarden

2007). Diabetic individuals exhibit impaired Akt activity regardless of expression levels (Krook, Roth et al. 1998). This affects both glucose transport and glycogen storage. The Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) pathway plays an important role in adipocyte differentiation and growth as well as β-oxidation (Kubota, Terauchi et al. 1999; Kadowaki 2000; Kadowaki, Hara et al. 2003). In the diabetic individual, PPAR-γ expression is defective (Barroso, Gurnell et al. 1999), leading to impaired lipid metabolism.

Obesity further contributes to the diabetic phenotype through dyslipidemia (Marette 2003). Excess adipose tissue increases circulatory Non-Esterified Fatty Acids (NEFA) (Kadowaki 2000; Shulman 2000; Inzucchi 2002) and pro-inflammatory cytokines such as Tumore Necrosis Factor Alpha (TNF-α) (Shulman 2000). NEFAs can further contribute to insulin resistance and hyperglycemia by increasing gluconeogenesis in the hepatocytes. Both NEFAs (Kadowaki 2000; Shulman 2000; Inzucchi 2002) and TNF-α (Kadowaki 2000; Marette 2003) can inhibiting IRSs which results in the impediment of the translocation of GLUT4 from the cytoplasm to the cellular membranes of myocytes.

Wu *et al* (Wu, Biggar et al. 2013) have published a thorough review of insulin resistance in hibernating squirrels from which he have adapted for our Figure 1.3B. In summary, hyperphagia is observed in the months prior to hibernation in order to increase metabolic fuel storage (i.e. adipose triglycerides).

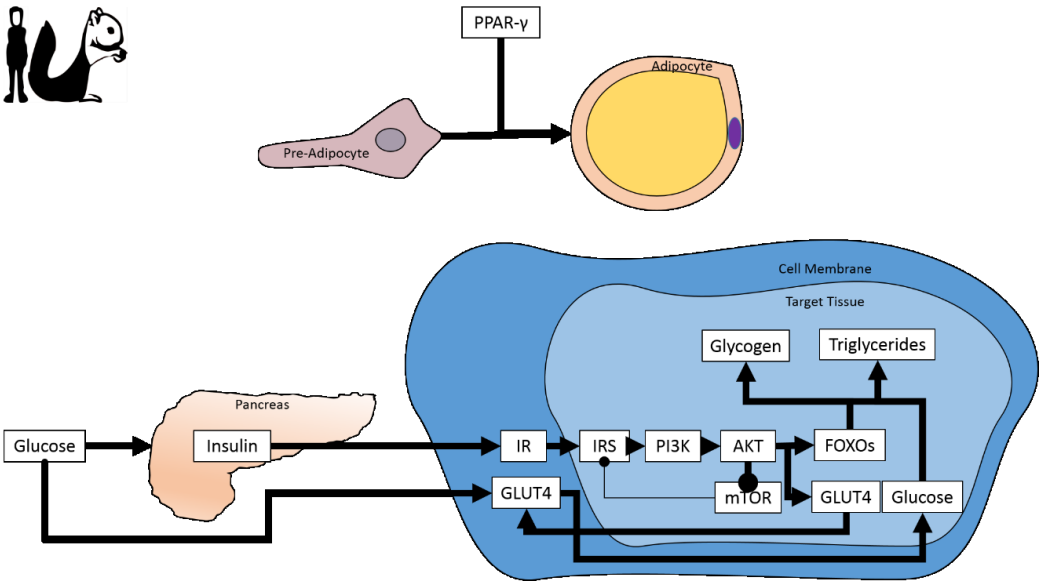
Hyperinsulinemia is observed in *Ictidomys spp*., with circulating insulin levels being 4 and 4.5 times higher than the summer active state in pre-hibernation and early hibernation, respectively. As hibernation progresses, insulin returns to basal levels, reversing the insulin resistant state (Figure 1.3C). The main mechanism involved in the reversibility of insulin resistance is an enhanced lipid metabolism. This is in contrast to diabetic individuals, who exhibit defective lipolysis.

The PI3K/Akt and PPAR-γ pathways seem to be the key players in the reversibility of insulin resistance in hibernators. Akt activity is diminished (Abnous, Dieni et al. 2008; McMullen and Hallenbeck 2010) while PPAR-γ expression is upregulated (Eddy, Morin et al. 2005) in torpid squirrels. Immediately upon arousal, both from hibernation in the early spring and during IBAs, Akt activity is enhanced (McMullen and Hallenbeck 2010). These changes suggest hibernators can readily shift their use in metabolic fuel by switching from carbohydrate to lipid metabolism throughout the hibernation season, exhibiting insulin resistance when fat metabolism is favored and heightening insulin sensitivity when glucose utilization is preferred.

It has been widely suggested that hibernating organisms should be used as models of reversible insulin resistance (Bloomgarden 2007; Martin 2008). However, to our knowledge there are no translational studies exploring the use of hibernation strategies for the treatment of obesity-induced insulin resistance and

type 2 diabetes. Currently, glycemic control is the standard of care in order to reduce the occurrence of diabetes (Inzucchi 2002).

A



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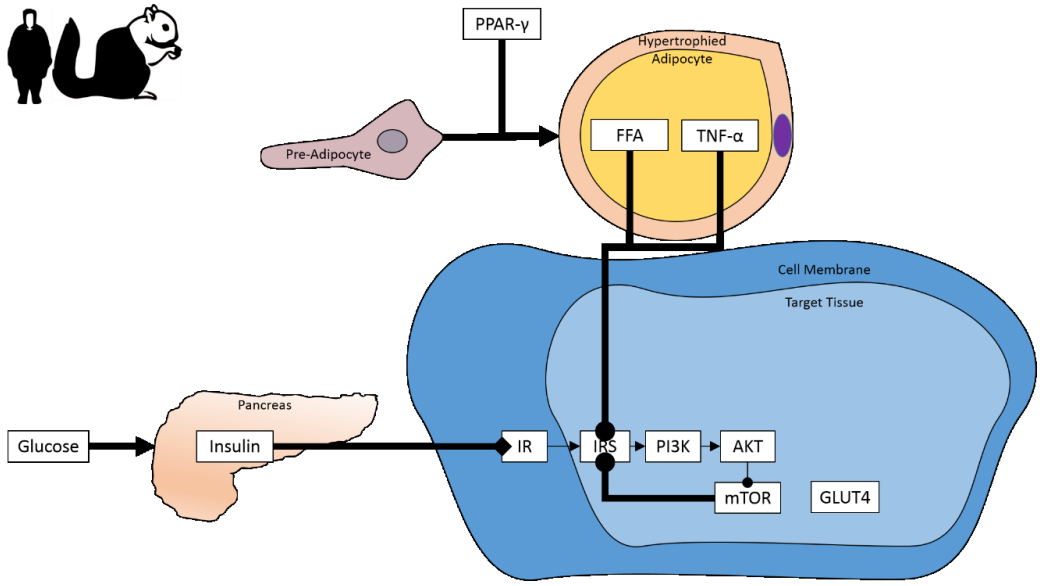

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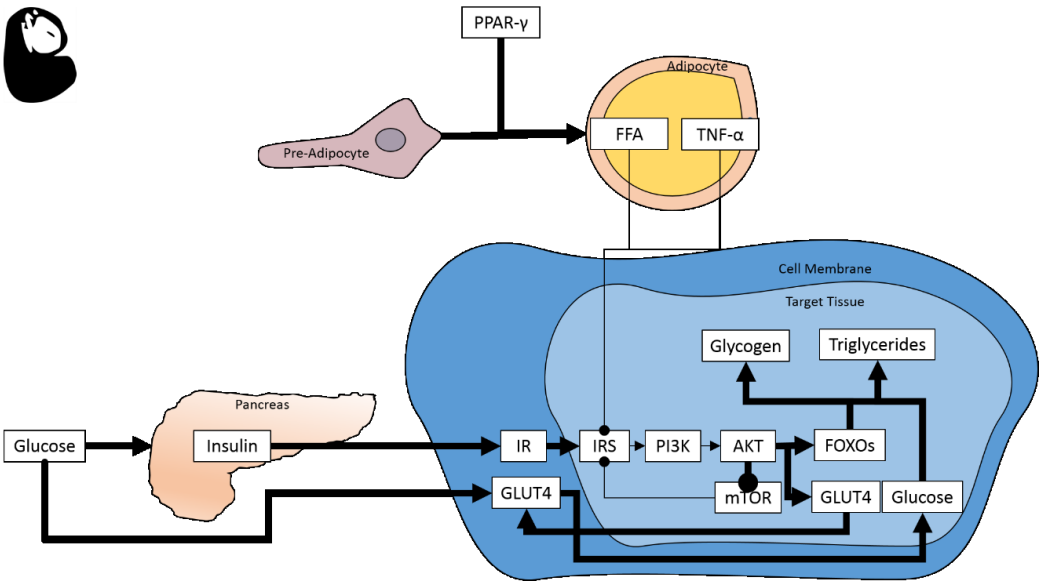


Figure 1.3. Insulin signaling.

In normal insulin signaling (A), increases in blood glucose stimulate insulin production which, through an interaction with its receptor in target tissues, promotes a cascade that results in the mobilization of GLUT4 to the cell membrane so that blood glucose is internalized by the cells and metabolized. During obesity-induced insulin resistance (B), hypertrophied adipocytes promote signals that interfere with glucose transport. Also, though insulin is produced, its receptor on the target tissues cannot respond to its signal, further interfering with glucose transport. Insulin resistance seems to be reversed (C) in hibernating ground squirrels. As their hypertrophied adipocytes reduce their size due to the use of fat storage as fuel source, the adipose signals that interfere with glucose transport lose strength. Furthermore, the target tissues regain their ability to respond to insulin signaling and glucose transport is restored. Line width represents the strength of the interaction. Arrowheads represent promotion. Circle heads represent inhibition. Diamond heads represent a resistance of the receptor to its substrate.

Organ Preservation

The only current treatment for end stage organ disease is transplantation (Jahania, Sanchez et al. 1999). In the 1970s, the prognosis for this procedure was limited by the immune reaction of the host body to the transplanted organ. As

immunosupressive drugs were developed and histocompatibility testing became available, organ rejection stopped being a limiting factor (Southard and Belzer 1995). Nowadays, tissue and organ transplantation is limited by their viability after storage.

There are two main techniques for organ preservation for transplantation 1) continuous/intermittent infusion and 2) cold static storage. Despite continuous infusion consistently proving to be the superior technique of the two, logistic and cost-associated constrains have made static storage the most widely used method for preserving organs (Mühlbacher, Langer et al. 1999; Rudd and Dobson 2009). Regardless of the technique, a storage solution is needed in order to perpetuate the viability of harvested organs. Currently, there are 167 storage solutions available in the US (Rudd and Dobson 2009) and none of them are considered an ideal medium (Southard and Belzer 1995). However, the University of Wisconsin (UW) solution is presently the "golden standard" for liver, kidney, and pancreas storage (Mühlbacher, Langer et al. 1999) as it is successful at keeping them viable for two days, three days, and three days, respectively (Southard and Belzer 1995). The true challenges for the available storage strategies are heart and lung, as neither is viable after 6 hours of cold static storage (Southard and Belzer 1995).

Presently, the mechanisms employed in preservation solutions for cold static storage rely on hypothermia to decrease metabolic activity, membrane

depolarization to reduce electrical and mechanical activity, impermeability to minimize cell swelling and extracellular edema, buffering to prevent acidosis, and antioxidants to limit reperfusion injury (Mühlbacher, Langer et al. 1999). There are a number of problems still unresolved with this approach such as Adenosine Triphosphate (ATP) depletion, ionic imbalance, and capillary collapse, among others (Southard and Belzer 1995). These issues limit tissue availability which in turn restricts the scope of organ transplantation.

A few groups have discovered the ability of bowel (Carey, Mangino et al. 2001), kidney (Green 1999), and liver (Lindell, Klahn et al. 2005) of hibernating animals to remain viable after extended periods of storage. However, those studies did not attempt to preserve organs from non-hibernating mammals borrowing from hibernation physiology; they only concluded that indeed the hibernation phenotype is more resistant to extended organ preservation. Most of the work done on the use of hibernation physiology for the preservation of organs for transplantation was done in the 1990s. In that time, much attention was given to what is now known as a Hibernation Induction Trigger (HIT) molecule – an 88 kDa opioid-like molecule capable of inducing torpidity in summer active hibernating species as well as in animals that do not naturally hibernate (Horton, Kaftani et al. 1998) first identified in 1969 (Dawe and Spurrier 1969). A few models of organ preservation using this HIT molecule (Oeltgen 1990; Chien, Oeltgen et al. 1991; Oeltgen, Horton et al. 1996; Bolling MD, Tramontini et al. 1997) and/or D-Ala2-D-Leu5-Enkephalin

(DADLE) (Oeltgen 1990; Oeltgen, Horton et al. 1996; Bolling MD, Tramontini et al. 1997; Bolling, Su et al. 1997), a supposed agonist, were developed, yet only one study conducted orthotopic transplantations (Oeltgen, Horton et al. 1996). In such study, lungs from dogs were successfully preserved with HIT-containing plasma from woodchucks for up to 33 hours and then transplanted with unaffected lung function. Nonetheless, all the experiments involving the HIT molecule are very controversial as its identification is regarded as an artifact (Dobson 2004). More contemporary experiments using hibernation strategies have not been particularly focused on extending preservation times but in completely changing the paradigm of organ transplantation by focusing on maintaining tissue viability in warm (Rudd and Dobson 2009) non-depolarizing conditions (Rudd and Dobson 2009; Rudd and Dobson 2011).

Hemorrhagic Shock

Every single person in the world is at risk of trauma regardless of social standing, race, religion, or political ideology. From an epidemiological perspective, in the United States, traumatic injury is the main cause of death amongst individuals ranging from the age of 1 to the age of 44. In the year 2000, this was attributable to the expenditure of $117 billion on medical care; 10% of the total US medical expenses. Worldwide, 5 million people had trauma-related deaths in 2000 (Kauvar and Wade 2005).

Hemorrhage is a problem of utmost importance in traumatic injury. Hemorrhagic shock makes bodily tissues become ischemic (Deitch 1992; Chatterjee, Patel et al. 2002; Kreimeier and Messmer 2002; Serafín, Roselló-Catafau et al. 2002; Powers, Zurawska et al. 2005; Fink, Hayes et al. 2008) and upon resuscitation aimed at the restoration of plasma volume, the reperfusion of tissues triggers an inflammatory cascade with subsequent organ failure and death (Deitch 1992; Yao, Redl et al. 1998; Rotstein 2000; Keel and Trentz 2005). The effective control of hemorrhage and the development of more efficient resuscitation strategies can save lives (Alam, Koustova et al. 2005).

By definition, hemorrhagic shock is the acute reduction of blood volume resulting in ischemia, the hypoperfusion of vital organs, and is evidenced by a dramatic reduction in cardiac output and arterial blood pressure. A consequence of hypoperfused tissues is a reduction in oxygen delivery so that aerobic metabolism can no longer be maintained, hence there is a shift to anaerobic metabolism. Anaerobic metabolism is not as efficient in ATP production as aerobic metabolism (Guyton and Hall 2001). Furthermore, during ischemia, ATP is catabolized to hypoxantine (Korthuis, Granger et al. 1985; Pacher, Nivorozhkin et al. 2006). If these conditions persist eventually ATP is depleted, resulting in cellular dysfunction and death.

Further complicating the ischemic event is the fact that the restoration of circulation after enduring low oxygen conditions results in oxidative stress known as reperfusion injury. Upon the reintroduction of normal oxygen levels, xanthine oxidase metabolizes hypoxantine into xanthine and then uric acid, generating superoxide and hydrogen peroxide (Korthuis, Granger et al. 1985; Pacher, Nivorozhkin et al. 2006). These ROS can cause direct injury to Deoxyribonucleic Acid (DNA) and proteins and induce apoptosis (Cooke, Evans et al. 2003; Sanada, Komuro et al. 2011). ROS can also open the mitochondrial permeability transition (MPT) pore (Kim, Jin et al. 2006) which can further reduce ATP production (Stavrovskaya and Kristal 2005).

Hemorrhagic Shock and Hibernation

Hibernators survive sluggish circulation followed by brief periods of increased heart rate and cardiac output during IBAs many times throughout the hibernation season without exhibiting the detrimental effects of ischemia (D'Alecy, Lundy et al. 1990) and reperfusion injury (Andrews 2007). Though the hibernating state is not accompanied by massive loss of blood, it exemplifies a remarkable physiological state characterized by a reduction in cardiac output and blood pressure comparable in magnitude to hemorrhagic shock. Furthermore, the classic hibernation patterns discussed earlier of cycles of torpidity scattered with IBAs closely resemble a number of ischemia/reperfusion events throughout the

hibernation season. Peculiarly, the brain and other tissues of hibernators are naturally shielded from ischemia (D'Alecy 1990) and reperfusion injury during arousal (Andrews 2007). Such protection is achieved by the employment of an array of inherent adaptations present in the hibernating animal (Graf and Schaller 2004).

Currently Available Resuscitative Strategies

Currently, resuscitation aims to restore intravascular volume and, in turn, blood pressure and systemic perfusion, preserving organ function. Taking into account both the hemorrhagic insult and the downfalls of resuscitation, the ideal resuscitative strategy would include the administration of a fluid that is effective at small infusion volumes, can be administered rapidly, has sustained effects, and does not cause further complications (Kyes and Johnson 2011).

Isotonic Resuscitation

Presently, the standard of care for hemorrhagic shock is based on studies performed between 1964 and 1975 by the groups of Shires (Shires, Coln et al. 1964; Shires, Carrico et al. 1970), Moyer (Dillon, Lynch Jr et al. 1966), and Moss (Cervera and Moss 1975). This guideline indicates the infusion of Lactated Ringer's (LR) in three times the shed blood volume. LR is effective at restoring blood pressure and increases cardiac output. However, large volumes are required which, as a result, do not allow for fast administration. Furthermore, since LR rapidly re-distributes through the extravascular space (Kyes and Johnson 2011),

continuous administration is required to observe sustained effects. Additionally, it can promote organ dysfunction and failure, boosting inflammation (Moore, McKinley et al. 2004). For these reasons, the clinical approaches for resuscitation are in continuous re-assessment.

Hypertonic Resuscitation

Hypertonic resuscitation was first explored in the 1960s but it was not until the 1980s when interest in this resuscitative strategy germinated. In fact, the term "small-volume resuscitation" was not forged until 1984 (Nakayama, Sibley et al. 1984) and the first human experiments were not conducted until 1987 (Holcroft, Vassar et al. 1987). The purpose of the administration of small-volume resuscitation fluids is primarily to treat trauma and shock patients (Kreimeier and Messmer 2002).

Despite the use of small-volume resuscitation fluids having proved beneficial in both the experimental and the clinical settings and their providing a more practical and sound strategy compared to the current standard of care (Kreimeier and Messmer 2002), they are still not being widely employed. Furthermore, there is no general agreement in the clinical setting as to which fluid therapy provides the most benefit (Angele, Schneider et al. 2008).

Hibernation-Based Resuscitation Fluids

Two research groups (Klein, Wendroth et al. 2010; Letson and Dobson 2011) are working on the development of hibernation-based resuscitation fluids for the treatment of hemorrhagic shock. Letson and Dobson (2011a; 2011b) have also borrowed from the hibernating animals to protect against ischemia-reperfusion injury by maintaining membrane potential (Dobson 2004). They have developed an additive for resuscitation that has been successful at improving survival both in rat (Letson and Dobson 2011; Letson and Dobson 2011) and pig (Granfeldt, Nielsen et al. 2012) models of hemorrhagic shock. In 2010, Klein et al (Klein, Wendroth et al. 2010) developed a small-volume resuscitation fluid. This fluid, called BHB/M, utilizing the hibernation protective strategies of hypothermia, use of an alternative fuel source, and increased antioxidant defense has proven effective in expanding the golden hour and achieving long-term (10 day) survival after blood return in a rat model of hemorrhagic shock. The same solution has been tested in a porcine model and a survival benefit was observed (Mulier, Lexcen et al. 2012).

BHB/M

The therapeutic effects of BHB/M can be attributable to many of its characteristics. For example, it is hyperosmolar, which probably increases plasma volume and organ perfusion (Nakayama, Sibley et al. 1984; Kreimeier, Brueckner et al. 1990; Krausz, Bar-Ziv et al. 1992; Kreimeier and Messmer 2002; Rocha-e-

Silva and Poli de Figueiredo 2005). Also, melatonin, one of its main components, is believed to scavenge reactive oxygen species generated during reperfusion (Zang, Cosma et al. 1998; Reiter, Tan et al. 2001; Tan, Manchester et al. 2007; Mathes, Kubulus et al. 2008). Furthermore, β-hydroxybutyrate, another main component, can reduce glucose utilization by serving as an alternative fuel source (Ide, Steinke et al. 1969; Krilowicz 1985; Isaad, Penicaud et al. 1987; Bartlett and Eaton 2004).

D-β-Hydroxybutyrate (BHB)

BHB is a ketone body, a by-product of fat metabolism. When hibernating mammals go through periods of inactivity, they rely on the energy stored in adipose tissue to fulfill their metabolic needs. This ketone body acts as an alternative fuel source by replacing glucose in energetic pathways, allowing the body to conserve energy, preserve cell function, and sustain life (Krilowicz 1985).

One of the main outcomes of hemorrhagic shock is ATP depletion which leads to cell death (Guyton and Hall 2001). BHB dehydrogenase can turn BHB into acetoacetate which can borrow a coenzyme-A (CoA) group from succinyl-CoA with the help of a transferase enzyme. Acetoacetate-CoA can then be turned into two acetyl-CoA molecules which can maintain ATP production (Kashiwaya, Takeshima et al. 2000). Hence, a single molecule of BHB is just as energy efficient as a molecule of glucose. This is particularly important in ischemic events since

glucose can only be converted to acetyl-CoA in aerobic conditions (Nelson, Lehninger et al. 2008).

Melatonin

Melatonin, a naturally-occurring pineal gland hormone, protects against injury by actively scavenging for pro-oxidative molecules, which can damage cell membranes and induce cell death, and modulating the immune response (Drew, Rice et al. 2001; Methes, Kubulus et al. 2008; Yang, Subeq et al. 2010). Hibernators have an almost 4-fold increase in endogenous melatonin levels during arousal from torpor compared to their active stages (Larkin, Yellon et al. 2003). Melatonin is a powerful antioxidant and free radical scavenger. Reperfusion of an ischemic cell generates reactive oxygen species (ROS), which can damage cell membranes, directly injure DNA and proteins, and exacerbate inflammatory processes (Cooke, Evans et al. 2003; Sanada, Komuro et al. 2011), all of which lead to apoptosis. Melatonin can neutralize ROS, minimizing reperfusion injury. Furthermore, the products of its reaction with free radicals, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), also possess antioxidant properties (Mayo, Sainz et al. 2005).

Dimethyl sulfoxide (DMSO)

Since melatonin is highly hydrophobic and difficult to get into solution, DMSO is used as a solvent. There is much controversy over its use, though. In

November 1965, a woman in Ireland, who was taking a variety of therapeutic agents, including DMSO, died of an allergic reaction. The Wall Street Journal published in December 1965 an article entitled “DMSO may have caused death of woman, makers of 'Wonder' drug warn doctors” (Carley 1965). In January 1966, the FDA banned clinical trials in the United States, mentioning the woman's death as a reason. Clinical research using DMSO was ceased and did not resume until 1972. However, its use is still contentious.

Objective

Only one concentration of each one of the components of BHB/M has been tested. For that reason, I operate under the hypothesis that enhancing the composition BHB/M will improve survival in a rat model of hemorrhagic shock. The objective of the work here presented is to optimize the composition and delivery of BHB/M. This objective will be achieved by a series of experiments in which both hypertonic and isotonic solutions will be administered.

In the hypertonic experiments, we will 1) evaluate two different modes of infusion and 2) conduct dose-ranging studies for the two main components of BHB/M: BHB and melatonin. A brief pilot study evaluating the hypothermia-promoting properties of 3-iodothyronamine (T1AM) will also be described. The isotonic experiments will be comprised of a comparison of a larger volume, lower molarity BHB/M to Lactated Ringer’s.

CHAPTER II: MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Charles River Laboratories International, Inc. (Portage, MI). They were housed in pairs in plastic cages, fed Laboratory Rodent Diet 5001 (LabDiet. St Louis, MO) ad libidum, provided with free access to water, and kept on a 12:12 light/dark cycle. All animals were allowed to acclimate to our Research Animal Resources facilities for at least three days before conducting experiments. The study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Anesthesia

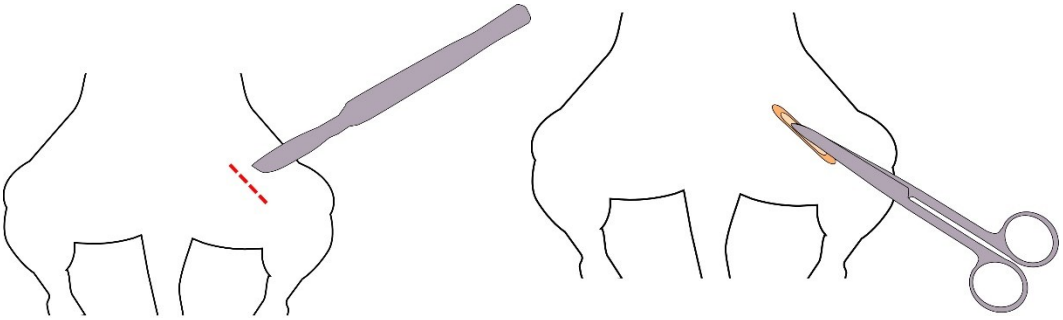
Rats were anesthetized with an inhaled anesthetic. Induction was conducted in an induction chamber with isoflurane (Clipper Distributing Company, LLC. St. Joseph, MO) at 5% in Breathing Grade Air (19.5%-23.5% Oxygen, ≤ 10 ppm Carbon Monoxide, ≤ 1000 ppm Carbon Dioxide, ≤ 24 ppm Water. Praxair Technology, Inc. Duluth, MN) at 1 l/min. Maintenance anesthesia was supported with ≤2% isoflurane, depending on individual requirements, through a rodent anesthesia mask. Both the induction chamber and the mask were connected to a vaporizer (Model 61020-SYS. Texas Scientific Instruments, LLC. Boerne, TX).

Surgical Cannulation

Animals were placed on supine position on a surgical board (Model 510-SS. Plas-Labs, Inc. Lansing, MI) and restrained with quick-tie nylon cords. After sterile preparation, a skin incision 1 cm in length was made on the left hind limb at the level of the adductor muscles (longus and magnus) 45 degrees to the mid line (Figure 2.1A). Then, fascia was removed (Figure 2.1B) and the femoral artery, vein, and nerve were exposed (Figure 2.1C) and separated using McPherson angled micro forceps (Model RS-5175. Roboz Surgical Instrument Co. Gaithersburg, MD) (Figure 2.1D). Vascular micro clips (Model RS-5424. Roboz Surgical Instrument Co. Gaithersburg, MD) were placed both proximally and distally on each vessel for tourniquet action (Figure 2.1E). Commercially available 24 GA I.V. catheters (Model 381412. BD Medical. Sandy, UT) were inserted into the left vein at a ~30 degree angle to initially perforate the vessel and then flattened to a position as close to a 0 degree angle as possible (Figure 2.1F). The catheter was furthered until it reached the proximally placed vascular clip. The previously tied piece of silk was slightly tightened to secure the catheter and the flash chamber was removed (Figure 2.1G). At this point, extension tubing was connected to the catheter. Vascular clips were removed, the catheter was advanced until fully inserted, and the silk tie was further tightened (Figure 2.1H). The left and right femoral arteries were prepared similarly. The left vein was employed for whole animal heparinization (10 IU) and

for fluid administration; the left artery, for blood pressure monitoring; and the right artery, for blood withdrawal.

A **B**



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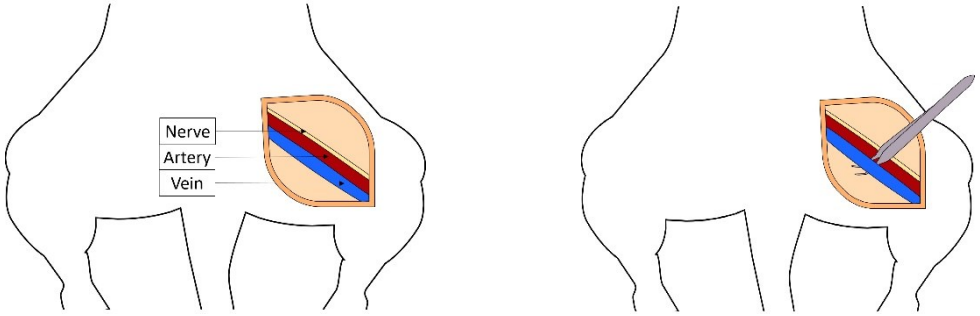


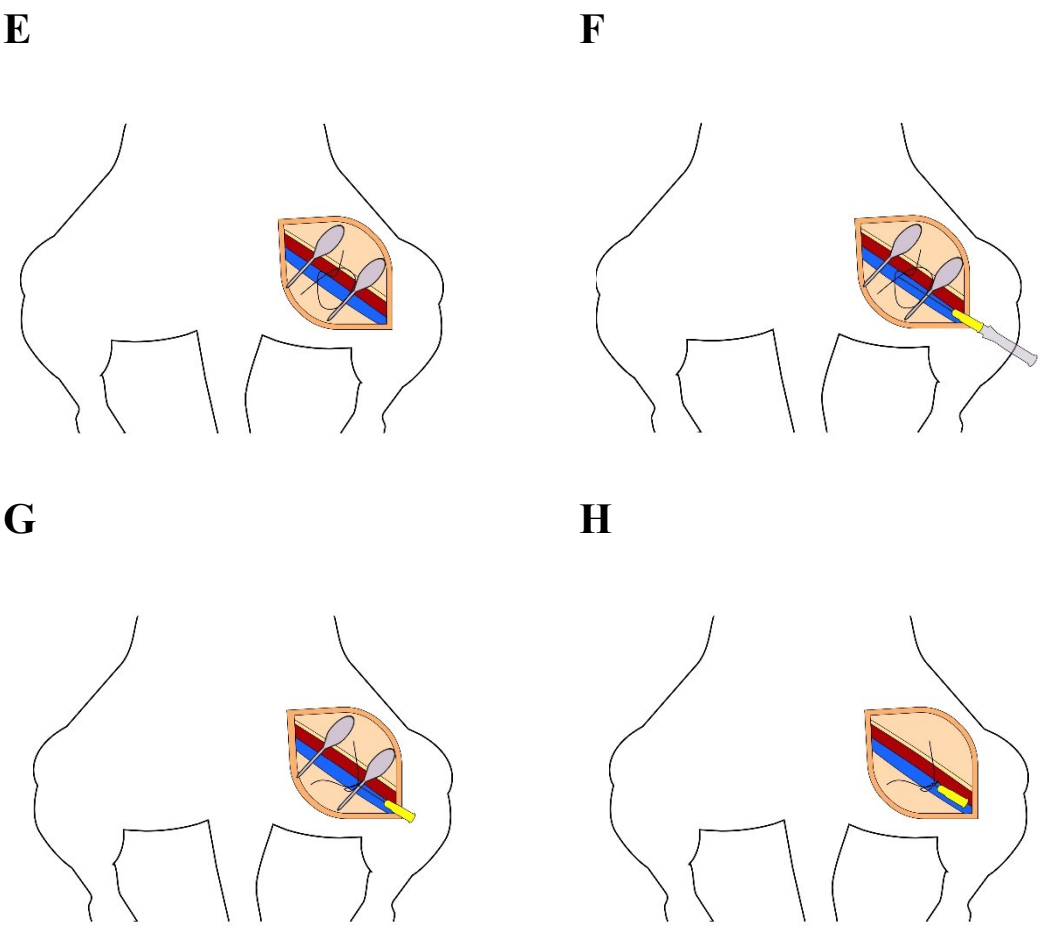


Figure 2.1. Surgical Cannulation Procedure.

(A) The hind limb is incised, (B) then the skin, subcutaneous tissue and muscle are separated, (C) allowing for visualization of the neurovascular package. (D) The nerve, vein, and artery are separated next. (E) Tourniquets and a tie for the cannula are placed, (F) leaving the vessel ready to be perforated with the cannula. (G) Then the cannula is secured and furthered and the needle, flash chamber, and safety chamber of the cannula are removed. (H) Finally, the tourniquets are removed.

Blood Volume Calculation

Blood volume (ml) was calculated as 6% of the body mass (g) plus 0.77 ml as per Lee and Blaufox (1985). This formula accounts for allometric differences in

blood volume as smaller animals have larger blood volumes relative to their size than larger animals.

Previously, Klein et al (2010) had used 6% of the body mass as the calculated blood volume. However, in the literature, the blood volume of the rat ranges anywhere from 6 to 10% of its body mass (Krinke, Bullock et al. 2000).

With our surgical procedure, we found that when 60% of the blood volume was removed using 6% of the body mass to calculate total blood volume, the hemorrhagic insult was not enough to ensure any deaths within 24 hours. However, when 7% of the body mass was used in the calculation, the hemorrhagic insult was too great and none of the animals survived particularly long. Using Lee and Blaufox's (1985) method resulted in nicely distributed survival/defunction times. Furthermore, using the 6% of the body mass plus 0.77 mL formula, the withdrawal of 40% of the calculated blood volume consistently resulted in a mean arterial blood pressure (MAP) of 25 mmHg (Appendix I)

Induction of Hemorrhagic Shock

Optimization of Delivery

Bolus Only Experiments

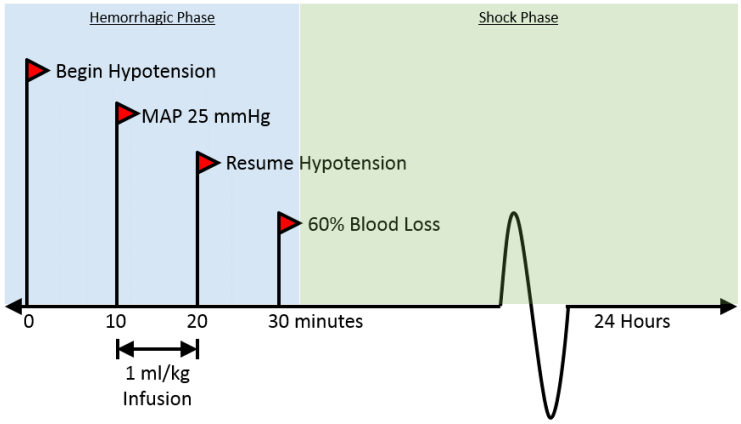
Following surgical cannulation, hypotension was achieved by reaching a mean arterial blood pressure (MAP) of 25 mmHg, which roughly corresponds to

the withdrawal of 40% of the calculated blood volume, over a 10-minute period. After the first hemorrhagic phase, a bolus infusion (1 ml/kg) of the published formulation of BHB/M was infused also over a 10-minute period. Hypotension was then resumed to achieve a total blood loss of 60% of the calculated blood volume. Blood samples were collected at four time points: 1) before hypotension (T0), 2) after 40% blood loss (T10), 3) 10 minutes after 40% blood loss (T20), and 4) after 60% blood loss (T30). Animals were monitored for 24 hours. A timeline of the hemorrhagic protocol is illustrated in Figure 2.2A.

Bolus Only plus Slow Infusion Experiments

Hemorrhagic shock was induced similarly to the Bolus Only protocol above, except that after the bolus administration and before resuming hypotension we initiated a slow infusion (100 µL/Kg) stage which was sustained for one hour. Blood samples were collected at five time points: 1) before hypotension (T0), 2) after 40% blood loss (T10), 3) 10 minutes after 40% blood loss (T20), 4) after 60% blood loss (T30), and 5) one hour after 60% blood loss (T90). This hemorrhagic shock protocol is depicted in Figure 2.2B.

A



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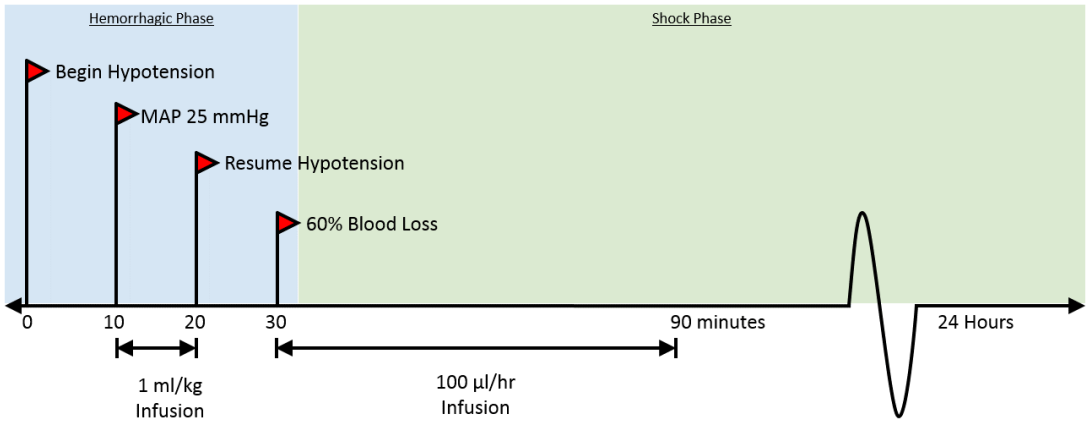


Figure 2.2 Optimization of delivery experiments: Experimental timeline.

After surgical preparation, animals were hemorrhaged until MAP ~25mmHg. They were then infused with either a single 1 ml/kg bolus (A) or a bolus followed by a 100 µL/hr slow infusion (B). In both instances, the bolus infusion was administered within 10 minutes. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume. No blood was transfused at any time point. Animals were monitored for 24 hours. All 24-hour survivors were euthanized.

Optimization of Composition

Hypertonic Resuscitation Experiments

The hemorrhagic shock protocol for all the experiments in this section was identical. It is very similar to the protocol described in the Bolus Only section above. It differs in that 50% of the shed blood was returned one hour after achieving 60% blood loss at a rate of 500 µl/min. Blood samples were collected at six time points: 1) before hypotension (T0), 2) after 40% blood loss (T10), 3) 10 minutes after 40% blood loss (T20), 4) after 60% blood loss (T30), 5) one hour after 60% blood loss (T90), and 6) after blood return (T105). Animals were monitored for 10 days. A timeline of this protocol is represented in Figure 2.3.

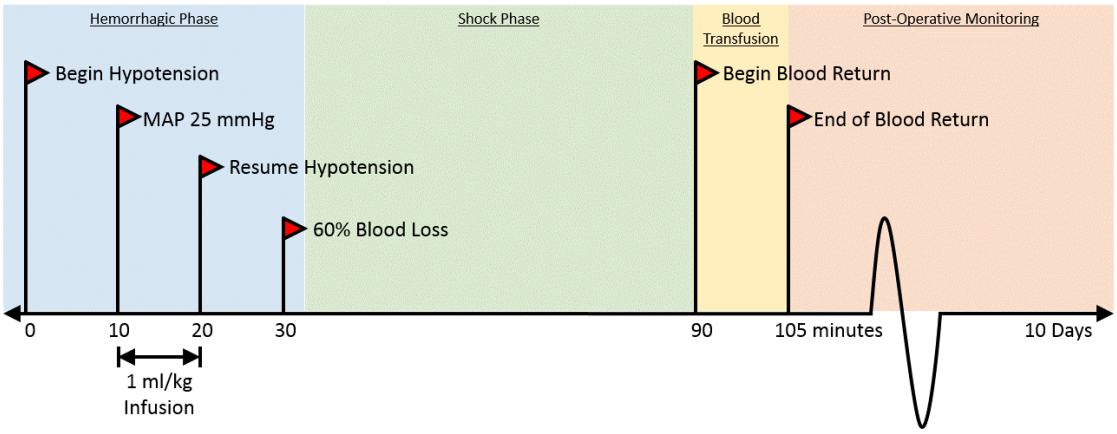


Figure 2.3. Hypertonic resuscitation experiments: Experimental timeline.

After surgical preparation, animals were hemorrhaged until MAP ~25mmHg and infused with a single 1 ml/kg bolus of solution over a 10 minute period. After bolus administration, animals were further hemorrhaged to 60% of their calculated blood volume and maintained in a shocked state for one hour. 50% of the shed blood volume was autotransfused at a rate of 500 µl/min 60 minutes after achieving 60% blood loss. Animals were monitored for 10 days. All 10-day survivors were euthanized.

Isotonic Resuscitation Experiments

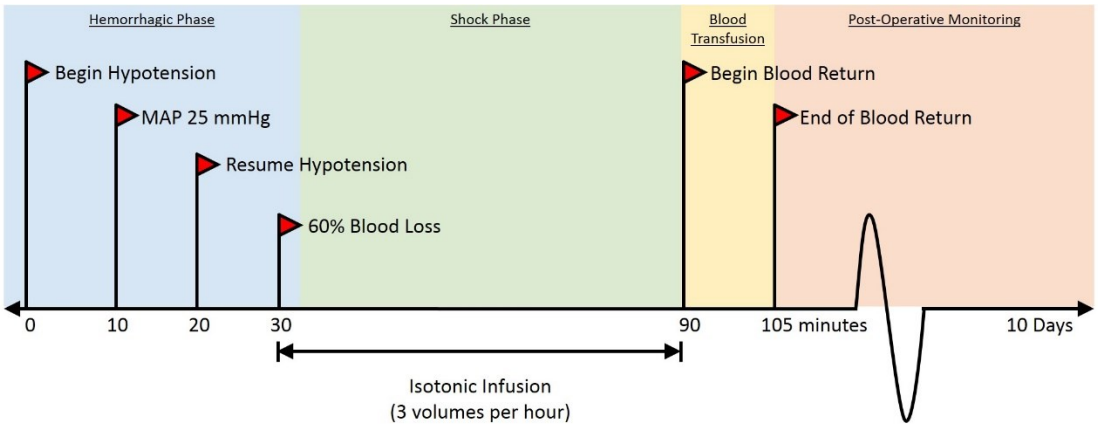
One-Hour Hemorrhagic Shock

Immediately after surgical instrumentation, blood was withdrawn via the right artery over 10 minutes until a MAP of 25 mmHg was obtained. Hypotension was paused for 10 minutes after which blood withdrawal resumed to achieve a total blood loss of 60% of the calculated blood volume. Immediately after achieving 60% blood loss, an infusion was started at a rate of 3 time the volume lost per hour for one hour. Immediately after, 50% of the shed blood was returned at a rate of 500 µl/min. The same six time point for blood sampling in the Hypertonic Resuscitation Experiments described above were used. Figure 2.4A represents the timeline for this protocol.

Three-Hour Hemorrhagic Shock

60% blood loss was achieved exactly as described for the One-hour Hemorrhagic Shock Protocol. Infusion of fluids was conducted at a rate of one time the volume lost per hour for three hours. Animals were then autotransfused 50% of their shed blood at 500 µl/min. Blood samples were collected at six time points: 1) before hypotension (T0), 2) after 40% blood loss (T10), 3) 10 minutes after 40% blood loss (T20), 4) after 60% blood loss (T30), 5) three hours after 60% blood loss (T210) and 6) after blood return (T225). Figure 2.4B shows this protocol's timeline.

A



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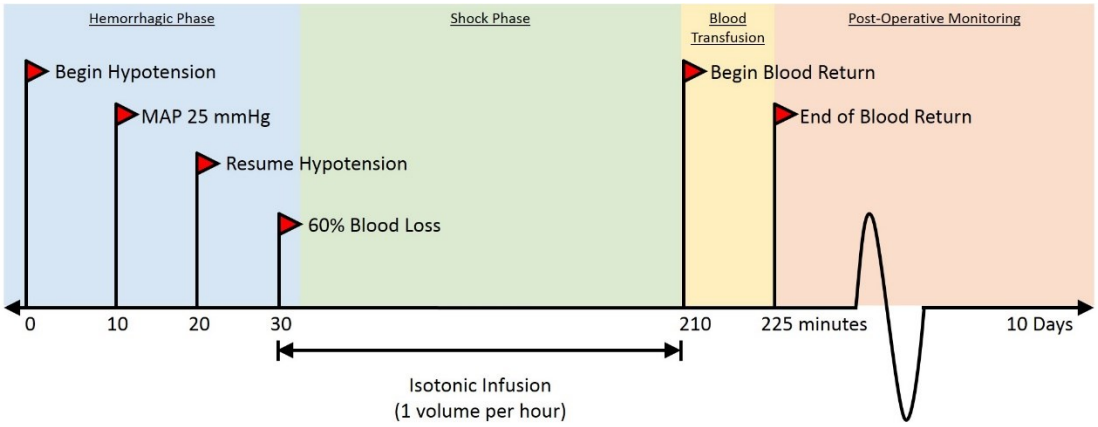

Figure 2.4. Isotonic resuscitation experiments: Experimental timeline.

After surgical preparation, animals were hemorrhaged until MAP ~25mmHg. After a 10 minute period, they were further hemorrhaged to 60% of their calculated blood volume. Then rats were administered isotonic solutions at a rate equal to (A) three volumes the shed blood per hour for an hour or (B) one volume the shed blood per hour for three hours. 50% of the shed blood volume was autotransfused at a rate of 500 µl/min either (A) 60 minutes or (B) 180 minutes after achieving 60% blood loss. Animals were monitored for 10 days. 10-day survivors were euthanized.

Wound Closure

After the hemorrhagic shock protocol came to a conclusion, all incisions were closed with a single cruciate stitch (internal cross) with 4-0 polypropylene suture (Prolene. Model 8521. Ethicon Endo-Surgery, Inc. Cincinnati, OH).

Experimental Treatments

All solutions were made fresh daily. The sodium salt form of the D-isomer of BHB was obtained from Ariel Pharmaceuticals, Inc. (Broomfield, CO). Melatonin was provided by Flamma S.p.A. (Bergamo, Italy). We used DMSO from Sigma-Aldrich Co. LLC. (St. Louis, MO).

Optimization of Composition

Hypertonic Resuscitation Experiments

Melatonin Dose-Ranging Study I

The published formulation of BHB/M containing 4 M BHB, 43 mM melatonin and 20% DMSO was compared against a solution containing 4 M BHB, 4.3 mM melatonin and 10% DMSO.

BHB Dose-Ranging Study

Solutions containing 4 M, 2 M, and 0.4 M BHB were compared. All solutions contained 4.3 mM melatonin and 10% DMSO.

Melatonin Dose-Ranging Study II

All solutions contained 4 M BHB and 2% DMSO. Melatonin was compared at concentrations of 4.3 mM, 0.43 mM, 0.0043 mM, 0.000043 mM, and 0 mM.

T1AM Pilot Study

Solutions containing T1AM at concentrations of 50 mg/kg, 25 mg/kg, 10 mg/kg, 2 mg/kg T1AM), or 0 mg/kg were compared in normotensive (not hemorrhaged) animals.

Isotonic Resuscitation Experiments

The solutions included in both the One-Hour and the Three-Hour Hemorrhagic Shock protocols and their compositions are described in Table 2.1. They are four: 1) LR, 2) LR plus 4.3 mM melatonin, 3) 140 mM BHB with 1.5x10-6 mM melatonin, and 4) 140 mM BHB with 4.3 mM melatonin.

Table 2.1. Isotonic resuscitation experimental design

Component	LR	LR / 4.3 mM Mel	140 mM BHB / $1.5x10^{-6}$ mM Mel	140 mM BHB / 4.3 mM Mel
3-D-β-hydroxybutyrate			140 mM	140 mM
D-L-lactate	28 mM	28 mM		
Sodium	130 mM	130 mM	140 mM	140 mM
Potassium	4 mM	4 mM		
Calcium	3 mM	3 mM		
Chloride	109 mM	109 mM		
Melatonin		4.3 mM	$1.5x10^{-6}$ mM	4.3 mM
DMSO		0.2%	0.02%	0.02%

Sample size n=10 for all treatment groups. Abbreviations: BHB-β-Hydroxybutyrate. DMSO-Dimethyl sulfoxide. LR- Lactated Ringer's. Mel-Melatonin.

Monitoring of Physiological Constants

Mean arterial blood pressure (MAP) and heart rate (HR) were recorded continuously via the left femoral artery cannula that was connected to a data acquisition device (PowerLab 4/30. ADInstruments, Inc. Colorado Springs, CO). Rectal temperature was continuously monitored using a rectal sensor.

Whole-Blood Parameters

Each blood sample was ran through a blood gas analyzer (Model ABL815 Flex. Radiometer America. Westlake, OH) for total hemoglobin (tHb), pH, saturation of oxygen (sO_2), potassium ion (K^+), sodium ion (Na^+), calcium ion (Ca^{++}), chloride ion (Cl^-), glucose (Glu), and lactate (Lac). For the Optimization of Delivery and Hypertonic Resuscitation Experiments, we also obtained partial pressure of oxygen (pO_2) and partial pressure of carbon dioxide (pCO_2).

Histopathological Scoring

10-day survivors were euthanized. Their tissues were harvested and fixed in 5% paraformaldehyde. We selected the brain, lung, and small intestine for histopathological assessment. The specific regions of each tissue are as follow: brain at Bregma ~-3, the left inferior lobe of the lung, and a mesenteric portion of the jejunum. Tissues were processed in a Model Tissue-Tek VIP 1000 tissue processor (Model 4617. Miles Laboratories Inc. Naperville, IL) (Appendix II).

They were embedded in paraffin using a Leica embedding center (Model EG1160. Leica Microsystems Inc. Buffalo Grove, IL). 5 μm sections were obtained with a manual microtome (Model 2030 Biocut. Reichert Inc. Munich, Germany) and mounted on StarFrost slides (Model CAS 8448WE. Mercedes Medical. Saratosa, FL). Slides were stained with hematoxylin and eosin (H&E) (Appendix III) with an autostainer (Model XL. Australian Biomedical Corporation Ltd. Melbourne, Australia). Slides were photographed at 20X magnification with an epifluorescence microscope (Model Eclipse 80i. Nikon Instruments Inc. Melville, NY) using SPOT Basic microscopy imaging software (Version 5.1. SPOT Imaging Solutions. Sterling Heights, MI). A scoring system on a 0-3 scale was developed (Table 2.2). This system was adapted from that used by Klein et al (2010) for brain, by Turkoglu et al (2012) for lung, and by Feinman et al (2010) for intestine. Three areas of the hippocampus (CA1, CA2, and CA3 neurons) and five random areas of the lung and intestine were examined. Sections were assessed blindly twice with a week in between observations and the scores were averaged.

Table 2.2. Histopathological scoring system.

		Lung	Intestine	Brain
0	No Evidence			
1	Mild	Alveolitis (2-3X); perivascular edema	Development of subepithelial Gruenhagen's space; vacuolization at the villus tip	Focal condensation of chromatin in the nucleus (pyknosis)
2	Moderate	Alveolitis (3-4X); interstitial edema	Lifting of epithelial layer from the lamina propria; increased vacuolization from the tip to midportion of villi.	Multifocal/diffuse pyknosis
3	Severe	Alveolitis (>5X); alveolar edema; inflammatory infiltrate; hemorrhage	Epithelial lifting and vacuolization from the tip to lower portion of villi; mucosal ulceration and disintegration of the lamina propria; inflammatory infiltrate; hemorrhage	Extensive pyknosis

Plasma Parameters

BHB (Appendix IV) and TNF-α (Appendix V) were measured using commercially available kits. For BHB we used a colorimetric assay (LiquiColor 2440. Stanbio Laboratories. Boerne, TX). TNF-α levels were determined using an Invitrogen immunoassay (Model KRC3011. Life Technologies. Carlsbad, CA). All reactions were conducted according to the manufacturers' manuals and read in the same absorbance microplate reader (SpectraMax Plus384. Molecular Devices, LLC. Sunnyvale, CA) using a data acquisition and analysis software (Softmax Pro version 5.4. MDS Analytical Technologies. Sunnyvale, CA). When appropriate, standard curves were generated and points were fit to a line using a curve fitting and data analysis software (CurveExpert Professional version 2.0.4. Hyams Development. Chattanooga, Tennessee).

Statistical Analyses

Survival curves were compared in SigmaPlot (version 11.0, Build 11.0.0.77) using a Gehan-Breslow-Wilcoxon test. Non-inferiority tests were also conducted in order to identify survival differences with the published formulation of BHB/M. One-way ANOVAs with Tukey's *post hoc* test were performed in JMP (version 8.0, Build 9200) to find differences between treatments for physiological constants, whole blood, and plasma data at each time point. Student's t-tests were used to compare 10-day survivors and non-survivors, regardless of treatment, within different time points for physiological constants, whole-blood, and plasma data. One-way ANOVAs were also used to establish histopathological score differences between treatments. A Cox Proportional Hazards regression model was conducted using a phreg procedure in SAS (version 9.4) to identify relationships between survival and physiological constants, whole-blood, and plasma data independently of treatment.

CHAPTER III: HYPERTONIC EXPERIMENTS

Survival

Optimization of Delivery

Klein et al's experiments (2010) administered BHB/M as a 1 ml/kg bolus followed by a 100 µl/hr slow infusion. We explored the possibility of administering BHB/M as a single bolus only. These experiments were conducted in acutely operated rats; these animals did not receive a blood transfusion 60% blood loss. No statistical differences ($p>0.05$) were observed in 24-hour survival when comparing the bolus only (mean survival 496.67 ± 314.59 min. n=6) to the bolus plus slow infusion (mean survival 149.20 ±142.71 min. n=5) protocol (Figure 3.1). For this reason, all further experiments were conducted administering a single 1 ml/kg bolus.

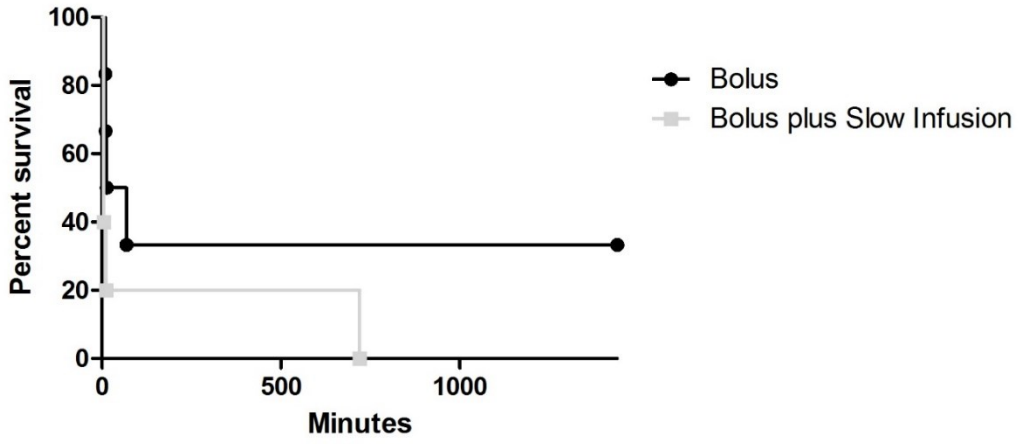

Figure 3.1. Optimization of delivery experiments: Kaplan-Meier plot of animals subjected to 60% blood loss.

Infusion of the published formulation of BHB/M was conducted either by administering either a single 1 ml/kg bolus (n=6) or a bolus followed by a 100 µL/hr slow infusion (n=5). The groups were not statistically different from each other. Times on the x-axis reflect minutes after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Optimization of Composition

Melatonin Dose-Ranging Study I

We decided to do a comparison between the published concentration of melatonin in BHB/M (43 mM), and a 10-fold decrease for two reason. Firstly, serum melatonin peaks in rats are ~8.61 × 10-7 mM (Benot, Molinero et al. 1998). Administering 43 mM melatonin at ml/kg would result in an overall bodily concentration of melatonin of .043 mM, almost fifty thousand times that of the peak physiological concentrations. Secondly, DMSO in this therapy acts simply as a solvent for melatonin and does not seem to provide any additional therapeutic benefits (Klein, Wendroth et al. 2010). Furthermore, its use is very controversial. A simple 10-fold decrease in the melatonin concentration administered would allow for DMSO to be lowered to 2% of the final volume. Hence, making our formulation less polemical than it needs to be.

The published formulation of BHB/M (n=6) was compared to a solution containing 4 M BHB, 4.3 mM melatonin and 10% DMSO (n=6). A group of animals (n=10) underwent a sham operation in which all the surgical procedures for femoral cannulation were conducted but the only blood drawn was for sampling (~6% blood loss).

Survival curves were compared at 24 hours and 10 days after 60% blood loss. 24-hour survival showed no statistical differences ($p>0.05$) between BHB/M (mean survival 21.00 ± 2.74 hrs) and 4 M BHB, 4.3 mM melatonin and 10% DMSO (mean survival 21.00 ± 2.74 hrs). There were also no statistical differences ($p>0.05$) in survival at 10 days (6.38 ± 2.00 days and 7.38 ± 1.75 days, respectively) (Figure 3.2; Table 3.1). All sham-operated rats lived to the experimental end point of 10 days.

Table 3.1. Melatonin Dose-Ranging Study I: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days.

Treatment		Mean ± SEM	
		24 Hrs	10 Days
4.3 mM Mel		21.00 ± 2.74	7.375 ± 1.75
43 mM Mel		21.00 ± 2.74	6.375 ± 2.00
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
4.3 mM Mel	43 mM Mel	1.0000	0.7980
4.3 mM Mel	Sham	0.1760	0.0143
43 mM Mel	Sham	0.1760	0.0143

Units are hours for the calculations at 24 hours (corresponding to Figure 3.2A) and days for the calculations at 10 days (corresponding to Figure 3.2B). The treatment labeled as 43 mM Mel contains 4 M BHB with 43 mM melatonin in 20% DMSO (n=6); the treatment labeled as 4.3 mM Mel contains 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel-melatonin.

BHB Dose-Ranging Study

After establishing that 4.3 mM melatonin had the same therapeutic effect as 43 mM melatonin, we explored the possibility of administering lower

concentrations of BHB. In these experiments, all treatments contained 4.3 mM melatonin and were dissolved in 2% DMSO.

BHB concentrations of 4 M (n=6), 2 M (n=5) and 0.4 M (n=5) were compared at 24 hours and 10 days after 60% blood loss (Figure 3.3). Table 3.2 summarizes pairwise comparison results. In short, at 24 hours, the 0.4 treatment had statistically lower survival (p<0.05) than the 4 M and the 2 M BHB treatments. At 10 days, only the difference between 0.4 M and 4 M BHB was upheld (p<0.05). However, 10-day mean survival showed a dose-dependent trend where the higher the concentration of BHB the longer the survival (4 M BHB, 7.38 ± 1.75 days; 2 M BHB, 5.25 ± 2.22 days; 0.4 M BHB, 2.07 ± 2.05 days). Consequently, all future experiments will contain BHB at a 4 M concentration.

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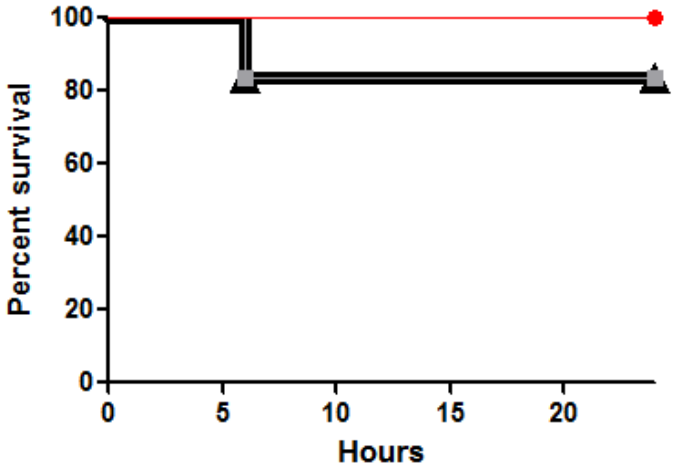

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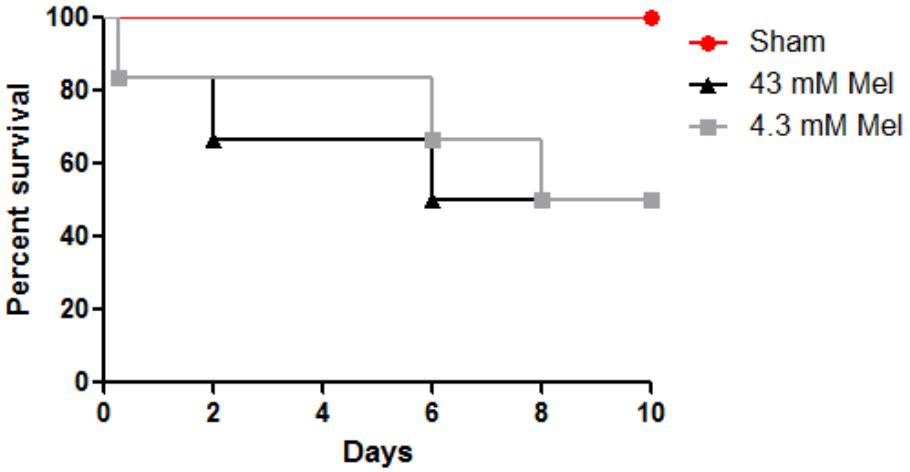

Figure 3.2. Melatonin Dose-Ranging Study I: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days.

Infusion of either 4 M BHB with 43 mM melatonin in 20% DMSO (n=6) or 4 M BHB with 4.3 mM melatonin in 10% DMSO (n=6) was achieved by administering a single 1 ml/kg bolus. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Non-Inferiority Test

Not observing statistical differences does not necessarily mean there are no practical or biologically relevant dissimilarities. Non-inferiority tests are designed to prove that one treatment is indistinguishable from another and their use has become a trend in the development of new therapies (Lesaffre 2008; Walker and Nowacki 2011). With this in mind, we conducted non-inferiority analyses to determine if the new formulations developed in this study are non-inferior to the published BHB/M. Non-inferiority was declared if the mean survival for each treatment was no worse than the mean survival for the published formulation of

BHB/M within a threshold difference (Δ) of two days. No treatments stayed within the specified boundary (Figure 3.4).

Table 3.2. BHB Dose-Ranging Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days.

Treatment		Mean ± SD	
		24 Hrs	10 Days
0.4 M BHB		6.50 ± 4.64	2.07 ± 2.05
2 M BHB		20.40 ± 3.22	5.25 ± 2.21
4 M BHB		21.00 ± 2.74	7.38 ± 1.75
Sham		24.00	10.00
Treatment Comparisons		*p* -value	
		24 Hrs	10 Days
0.4 M BHB	2 M BHB	0.0398	0.0803
0.4 M BHB	4 M BHB	0.0222	0.0472
0.4 M BHB	Sham	0.0004	0.0004
2 M BHB	4 M BHB	0.8920	0.4990
2 M BHB	Sham	0.1380	0.0041
4 M BHB	Sham	0.1760	0.0102

Units are hours for the calculations at 24 hours (corresponding to Figure 3.3A) and days for the calculations at 10 days (corresponding to Figure 3.3B). Sample sizes are as follows: 4 M BHB, n=6; 2 M BHB, n=5; 0.4 M BHB, n=5. All solutions contained 4.3 mM melatonin and 10% DMSO. Statistically significant *p*-values are colored in red and underlined. Abbreviations: BHB-β-Hydroxybutyrate.

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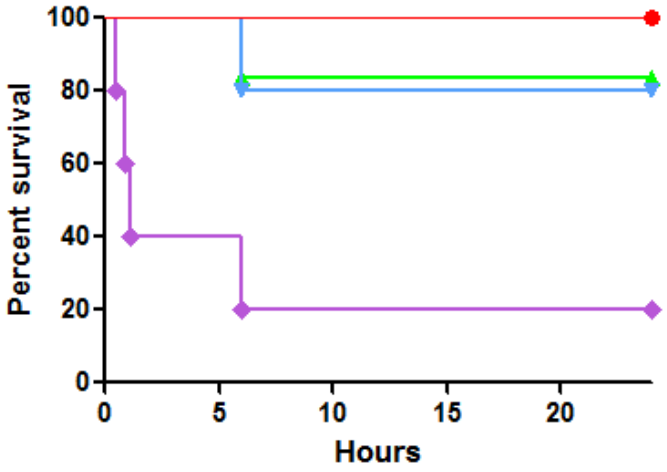

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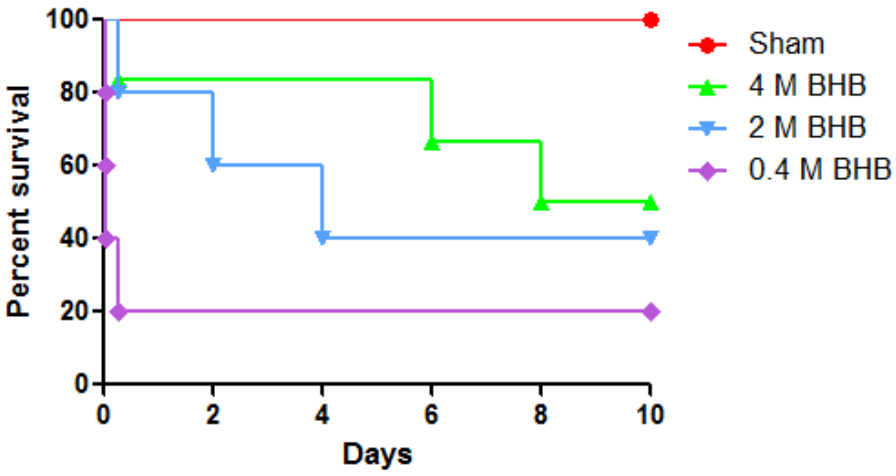

Figure 3.3. BHB Dose-Ranging Study: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days.

Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Sham-operated animals are also included in the graph. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Survival curves for the concentrations of melatonin, depicted in Figure 3.5, were compared 24 hours and 10 days after 60% blood loss. Pairwise comparisons are summarized in Table 3.3. No treatment differences were observed at either 24 hours or 10 days after 60% blood loss. However, at 10 days, only the treatments with 0 mM melatonin (4.38 ± 1.42 days), 0.0043 mM Mel (6.58 ± 1.60 days), and the NaCl control (4.58 ± 1.42 days) were different (p<0.05) from the sham group.

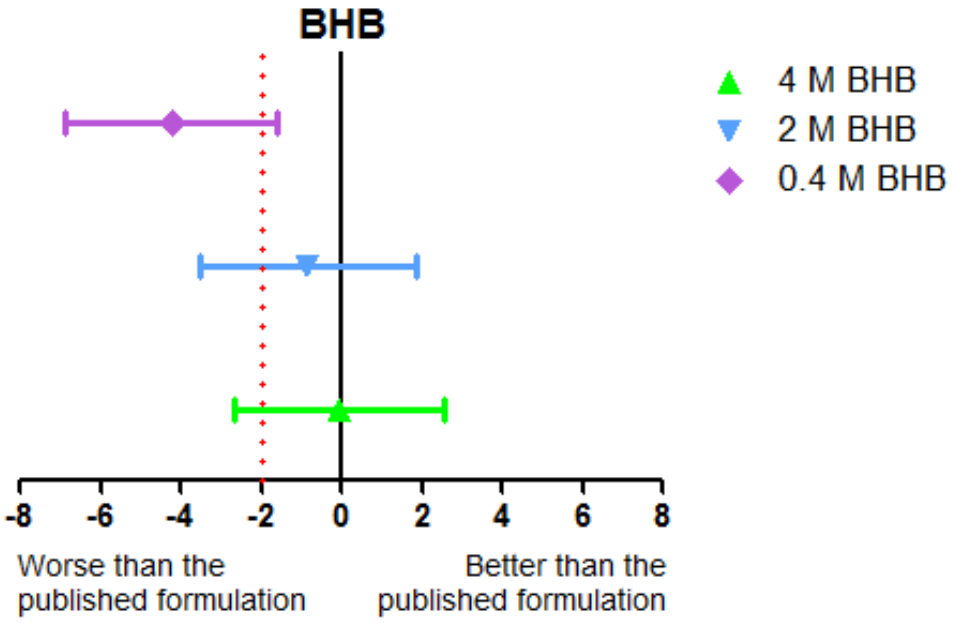

Figure 3.4. BHB Dose-Ranging Study: Non-inferiority plot of animals subjected to 60% blood loss at 10 days.

Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), 0.4 M BHB (n=5), or the published formulation of BHB/M (n=8) was achieved by administering a single 1 ml/kg bolus. The published formulation of BHB/M contained 4 M BHB, 43 mM melatonin, and 20% DMSO. All other solutions contained 4.3 mM melatonin and 10% DMSO. Times on the x-axis reflect differences in survival times in days after achieving 60% blood loss. Units are expressed as mean survival in days ± 95% Confidence Interval

Non-Inferiority Test

We considered a treatment to be non-inferior to the published formulation of BHB/M if its mean survival was no worse than that for BHB/M within a Δ of two days (Figure 3.6). All combinations of BHB and melatonin stayed within the two-day boundary established in order to determine non-inferiority. Only BHB alone and the NaCl control were found inferior to the published BHB/M.

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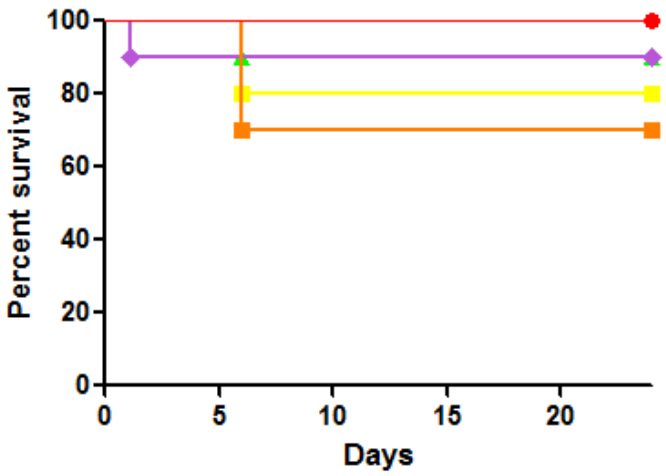

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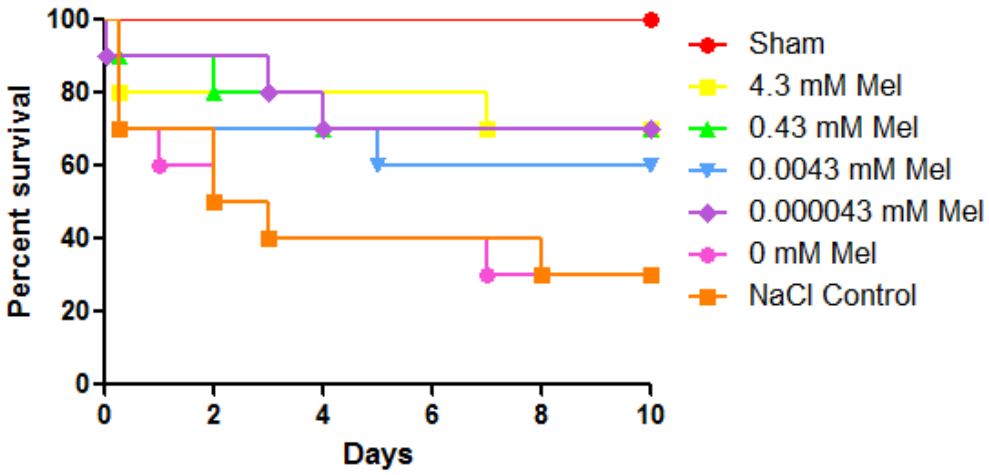

Figure 3.5. Melatonin Dose-Ranging Study II: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days.

Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Sham-operated animals are also included in the graph. The Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Physiological Constants

MAP, HR and rectal temperature were monitored in real time throughout the hemorrhagic protocol. Only experiments where survival differences between hemorrhaged groups were observed will be described in this section, with the exception of the T1AM pilot study in which depressed body temperature was the only parameter monitored.

Table 3.3. Melatonin Dose-Ranging Study II: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days.

Treatment		Mean ± SEM	
		24 Hrs	10 Days
0 mM Mel		18.6 ± 3.20	4.38 ± 1.42
0.000043 mM Mel		21.71 ± 2.76	7.71 ± 1.10
0.0043 mM Mel		18.60 ± 3.20	3.58 ± 1.60
0.43 mM Mel		22.20 ± 2.30	7.63 ± 1.44
4.3 mM Mel		20.40 ± 3.22	7.75 ± 1.49
NaCl Control		18.60 ± 3.20	4.58 ± 1.42
Sham		24	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
Sham	0 mM Mel	0.0555	0.0009
Sham	4.3 mM Mel	0.1280	0.0555
Sham	0.43 mM Mel	0.2940	0.0555
Sham	0.0043 mM Mel	0.0555	0.0229
Sham	0.000043 mM Mel	0.2940	0.0555
Sham	NaCl Control	0.0555	0.0009
0 mM Mel	4.3 mM Mel	0.6150	0.1200
0 mM Mel	0.43 mM Mel	0.2760	0.0792
0 mM Mel	0.0043 mM Mel	1.0000	0.3560
0 mM Mel	0.000043 mM Mel	0.3570	0.0864
0 mM Mel	NaCl Control	1.0000	0.9080
4.3 mM Mel	0.43 mM Mel	0.5420	0.9630
4.3 mM Mel	0.0043 mM Mel	0.6150	0.5910
4.3 mM Mel	0.000043 mM Mel	0.6260	0.9630
4.3 mM Mel	NaCl Control	0.6150	0.1310
0.43 mM Mel	0.0043 mM Mel	0.2760	0.5630
0.43 mM Mel	0.000043 mM Mel	0.9420	1.0000
0.43 mM Mel	NaCl Control	0.2760	0.0866
0.0043 mM Mel	0.000043 mM Mel	0.3570	0.6570
0.0043 mM Mel	NaCl Control	1.0000	0.3550
0.000043 mM Mel	NaCl Control	0.3570	0.0866

Units are hours for the calculations at 24 hours and days for the calculations at 10 days. Sample sizes are as follows: 4.3 mM melatonin, n=10; 0.43 mM melatonin, n=10; 0.0043 mM melatonin, n=10); 0.000043 mM melatonin, n=10; and 0 mM melatonin, n=10. These solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Statistically significant *p*-values are colored in red and underlined. Abbreviations: Mel-melatonin.

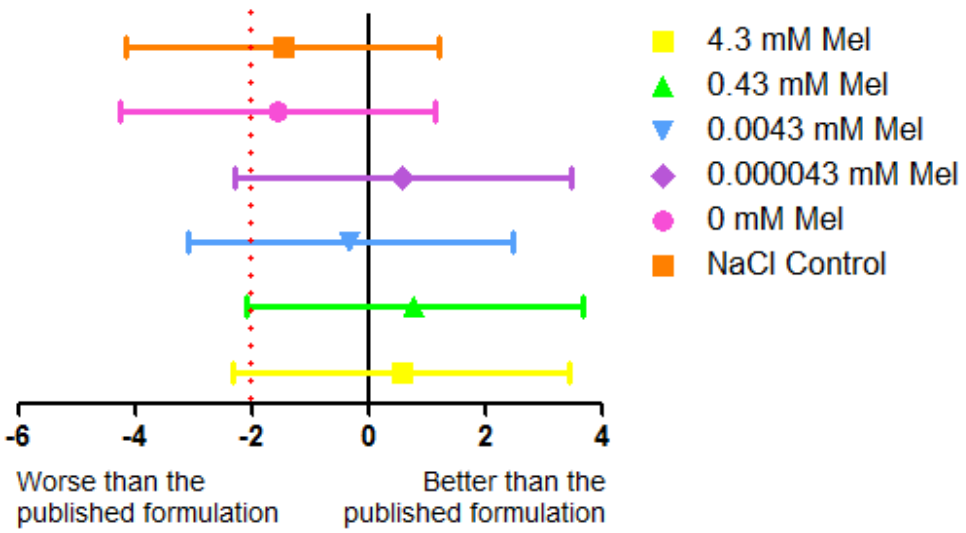

Figure 3.6. Melatonin Dose-Ranging Study II: Non-inferiority plot of animals subjected to 60% blood loss at 10 days.

Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). All of the aforementioned treatments are being compared to the published formulation of BHB/M (n=8) containing 4 M BHB, 43 mM melatonin, and 20% DMSO. Times on the x-axis reflect differences in survival times in days after achieving 60% blood loss. Units are expressed as mean survival in days ± 95% Confidence Interval. Dashed line represents a threshold difference (Δ) of two days.

Optimization of Composition

BHB Dose-Ranging Study

Table 3.4 and Figure 3.7 compile differences between treatments.

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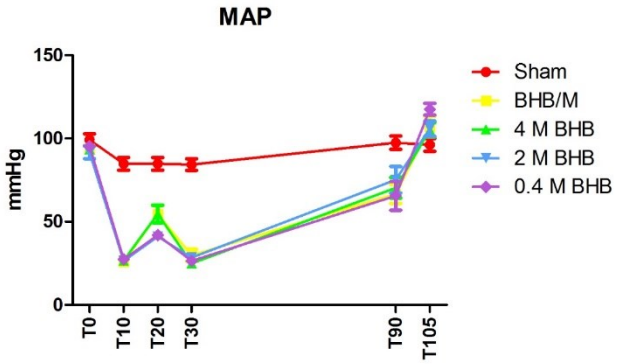

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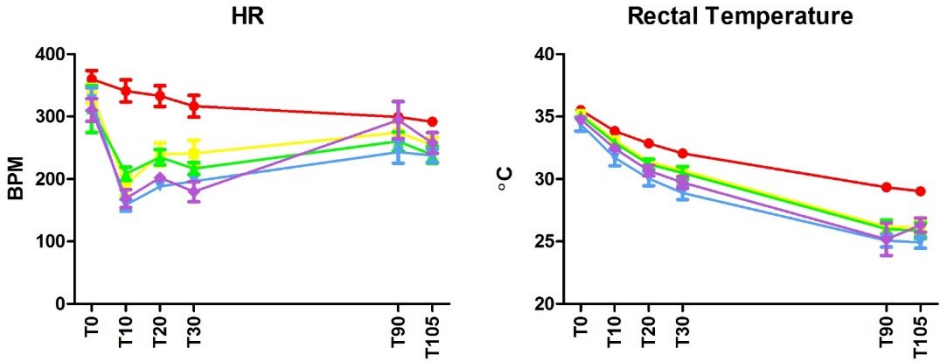


Figure 3.7. BHB Dose-Ranging Study: Physiological constant data.

Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Table 3.4. BHB Dose-Ranging Study: Physiological constant data.

MAP (mmHg)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	99.20 ± 12.00	A	84.77 ± 12.57	A	84.77 ± 12.57	A	84.29 ± 11.70	A	97.45 ± 13.33	A	96.18 ± 12.66	A
4 M BHB	93.38 ± 7.90	A	26.66 ± 3.06	B	54.66 ± 15.69	B	24.94 ± 2.50	B	70.35 ± 18.55	B	105.22 ± 12.78	A
2 M BHB	91.56 ± 9.22	A	26.47 ± 0.66	B	41.01 ± 5.42	B	28.42 ± 5.75	B	75.22 ± 19.64	AB	105.89 ± 11.19	A
0.4 M BHB	95.29 ± 5.03	A	27.27 ± 1.91	B	41.92 ± 10.41	B	26.38 ± 4.27	B	65.57 ± 22.71	B	117.56 ± 9.35	A
HR (BPM)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	360.32 ± 42.95	A	341.18 ± 56.17	A	333.03 ± 51.95	A	316.69 ± 55.00	A	299.54 ± 20.15	A	291.76 ± 23.90	A
4 M BHB	312.20 ± 112.75	A	208.35 ± 32.46	B	234.86 ± 36.97	B	216.84 ± 28.65	B	260.40 ± 45.33	AB	238.98 ± 40.42	B
2 M BHB	327.85 ± 48.80	A	158.39 ± 24.34	B	188.24 ± 18.09	B	196.41 ± 13.86	B	243.12 ± 46.64	B	237.73 ± 27.70	B
0.4 M BHB	310.76 ± 44.63	A	168.72 ± 35.32	B	202.13 ± 14.96	B	179.89 ±39.96	B	294.55 ± 59.50	AB	257.81 ± 29.07	AB
Rectal Temperature (°C)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	35.52 ± 0.37	A	33.83 ±0.48	A	32.86 ± 0.67	A	32.06 ± 0.65	A	29.35 ± 0.56	A	29.03 ± 0.54	A
4 M BHB	35.10 ± 0.62	AB	32.92 ± 1.05	AB	31.19 ± 1.20	B	30.48 ± 1.55	BC	26.01 ± 2.13	B	25.85 ± 1.86	B
2 M BHB	34.40 ± 1.47	B	31.70 ± 1.65	B	30.04 ± 1.53	B	28.89 ± 1.42	C	25.07 ± 1.36	B	24.93 ±1.25	B
0.4 M BHB	34.79 ± 0.73	AB	32.45 ± 0.73	AB	30.67 ± 1.06	B	29.73 ± 1.16	BC	25.18 ± 2.61	B	26.31 ± 0.98	B

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: BHB-β-Hydroxybutyrate.

MAP provides information about systemic perfusion. MAP was higher in shams at T10, T20, and T30 ($p<0.0001$) compared to all hemorrhaged groups. This is to be expected as shams only had blood drawn for sampling. At T90, all hemorrhaged groups were 20-30 mmHg lower than sham-operated animals. However only the published formulation of BHB/M, the treatment with 4 M BHB, and the treatment with 0.4 M BHB showed statistical differences ($p<0.05$) when compared to shams.

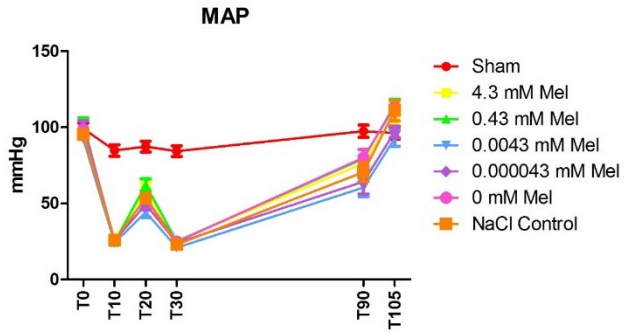
HR is an important player in determining cardiac output (CO) and hence heart function, blood transport, and oxygen delivery. Shams had higher HR than all other treatments at T10 ($p<0.0001$), T20 ($p<0.0001$), and T30 ($p<0.01$). By T90, hemorrhaged animals had a HR close to that of shams.

Monitoring rectal temperature is important for cerebral metabolic rate drop 6 to 10% per 1°C reduction in body temperature, decreasing glucose utilization and oxygen consumption which are significant protective effects of hypothermia (Polderman 2009). All animals presented a gradual decrease in rectal temperature as a result of anesthesia. However, the reduction in hemorrhaged animals was sharper than in shams.

Melatonin Dose-Ranging Study II

Treatment differences can be observed in Table 3.5 and Figure 3.8.

A



B **C**

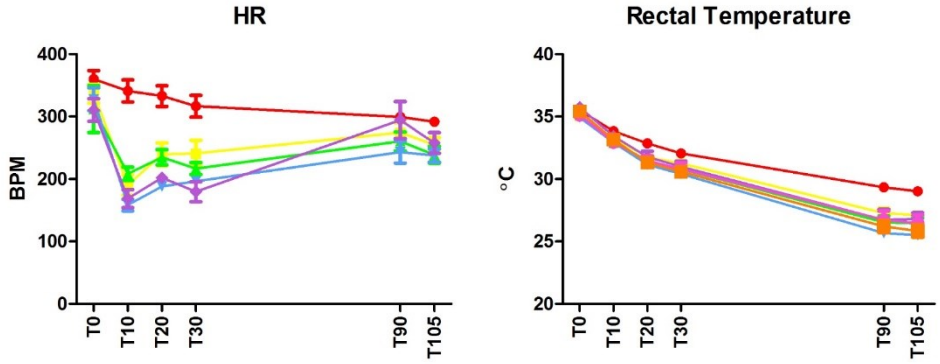


Figure 3.8. Melatonin Dose-Ranging Study II: Physiological constant data.

Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

MAP was lower in all treatments compared to shams (p<0.0001) through the blood withdrawal phase. During the one-hour hemorrhage period MAP rose

steadily to levels close to those of shams. After a blood transfusion, MAP reached levels higher than baseline.

Sham-operated animals had higher HR than all hemorrhaged groups through the experiment, with T0 being the exception.

All groups, including shams, had gradual decreases in rectal temperature throughout the experimental protocol. However, sham-operated animals consistently showed rectal temperatures a few degrees above all hemorrhaged animals.

Sham rats were excluded when comparing animals that survived to 10 days after 60% blood loss and those that did not because, since shams were not hemorrhaged and they all lived to 10 days, they could skew the results. MAP, HR, and rectal temperature were higher at T90 in animals that lived to 10 days ($p<0.05$). HR was also higher at T105 ($p<0.05$) in 10-day survivors.

Shams were not included in out Cox Proportional Hazards regression analyses also. At T90, lower MAP ($p<0.01$; HR=0.97), HR ($p<0.05$; HR=0.99), and rectal temperature ($p<0.05$; HR=.49) resulted in lower survival. Higher rectal temperature ($p<0.05$; HR=2.29) at T20 lead to a decrease in survival.

Table 3.5. Melatonin Dose-Ranging Study II: Physiological constant data.

MAP (mmHg)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	99.20 ± 12.00	A	84.77 ± 12.57	A	84.77 ± 12.57	A	84.29 ± 11.70	A	97.45 ± 13.33	A	96.18 ± 12.66	AB
4.3 mM Mel	97.31 ± 6.15	A	25.97 ± 0.96	B	58.84 ± 14.20	B	25.48 ± 2.79	B	76.28 ± 17.66	AB	108.71 ± 17.23	AB
0.43 mM Mel	102.56 ± 10.91	A	24.59 ± 0.88	B	62.22 ± 11.77	B	25.10 ± 4.67	B	79.39 ± 8.39	AB	114.86 ± 10.57	A
0.0043 mM Mel	92.15 ± 8.60	A	24.08 ± 1.56	B	44.30 ± 11.26	C	20.89 ± 3.81	B	60.49 ± 20.53	B	92.86 ± 17.61	B
0.000043 mM Mel	100.62 ± 10.62	A	25.57 ± 1.78	B	49.28 ± 9.84	BC	24.07 ± 6.60	B	64.35 ± 23.16	B	97.07 ± 9.86	AB
0 mM Mel	97.92 ± 5.76	A	25.75 ± 0.99	B	53.74 ± 7.78	BC	24.86 ± 2.70	B	80.06 ± 16.95	AB	113.65 ± 9.35	A
NaCl Control	95.21 ±3.65	A	25.90 ± 2.27	B	53.29 ± 7.55	BC	22.91 ± 3.17	B	70.60 ± 19.50	B	111.06 ± 21.17	AB
HR (BPM)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	367.09 ± 46.53	A	348.23 ± 58.20	A	338.40 ± 52.39	A	321.71 ± 54.76	A	303.60 ± 23.39	A	295.79 ± 26.31	A
4.3 mM Mel	354.19 ± 34.16	A	213.71 ± 46.85	B	256.07 ± 48.12	B	215.24 ± 52.11	B	278.12 ± 29.04	AB	258.46 ± 24.13	AB
0.43 mM Mel	377.49 ± 57.74	A	193.78 ± 44.04	B	255.21 ± 35.59	B	215.07 ± 28.74	B	260.44 ± 23.45	AB	251.39 ± 24.88	B
0.0043 mM Mel	364.37 ± 36.37	A	217.33 ± 36.05	B	219.63 ± 34.44	B	229.02 ± 64.67	B	241.31 ± 52.85	B	221.89 ± 51.64	B
0.000043 mM Mel	363.69 ± 23.42	A	210.02 ± 17.45	B	220.20 ± 19.06	B	227.77 ± 57.98	B	254.37 ± 45.95	AB	229.09 ± 28.01	B
0 mM Mel	366.59 ± 48.82	A	214.83 ± 72.93	B	240.45 ± 32.60	B	225.22 ± 29.28	B	271.36 ± 35.08	AB	251.02 ± 23.16	B
NaCl Control	342.91 ± 26.68	A	195.29 ± 31.76	B	215.62 ± 23.80	B	201.76 ± 29.35	B	244.42 ± 31.59	B	230.66 ± 27.16	B
Rectal Temperature (°C)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	35.52 ± 0.37	A	33.83 ± 0.48	A	32.86 ± 0.67	A	32.06 ± 0.65	A	29.35 ± 0.56	A	29.03 ± 0.54	A
4.3 mM Mel	35.05 ± 0.66	A	33.27 ± 0.83	A	31.75 ± 0.82	AB	31.26 ± 0.86	AB	27.27 ± 1.33	AB	27.11 ± 1.07	B
0.43 mM Mel	35.13 ± 0.49	A	32.90 ± 0.88	A	31.36 ± 1.04	B	30.79 ± 1.12	AB	26.55 ± 1.56	B	26.46 ± 1.28	B
0.0043 mM Mel	34.92 ± 0.65	A	32.92 ± 0.72	A	31.16 ± 0.83	B	30.41 ± 0.88	B	25.67 ± 1.11	B	25.50 ± 1.26	B
0.000043 mM Mel	35.74 ± 0.70	A	33.45 ± 0.95	A	31.78 ± 1.27	AB	30.99 ± 1.28	AB	26.73 ± 2.39	B	26.81 ± 1.34	B
0 mM Mel	35.15 ± 0.48	A	32.96 ± 1.22	A	31.47 ± 1.29	B	30.81 ± 1.59	AB	26.75 ± 2.18	B	26.46 ± 2.04	B
NaCl Control	35.42 ± 0.64	A	33.16 ± 0.69	A	31.32 ± 0.87	B	30.59 ± 1.14	B	26.21 ± 1.56	B	25.84 ± 1.65	B

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: Mel-Melatonin.

T1AM Pilot Study

3-iodothyronamine (T1AM) has been shown to induce non-shivering hypothermia in mice (Scanlan, Suchland et al. 2004). For that reason, we hypothesized that T1AM, in combination with the optimized BHB/M can induce a hibernation-like protective state that improves recovery from normally lethal hemorrhagic shock.

Initially, we decided to investigate whether the observations made by Scanlan et al (2004) could be replicated in a rat model. Therefore, we conducted a pilot study to determine whether T1AM could induce hypothermia in normotensive animals subjected to the instrumentation procedures common to our hemorrhagic shock model. None of these animals were hemorrhaged. The treatments included a semi-log dose range from 0 to 50 mg/kg in 10 rats (n=2 per treatment). Though a reduction in temperature was observed, there were no observable differences between treatments (Figure 3.9). It is possible that the anesthesia could be masking the thyroid hormone derivative. It has been described that mice injected with T1AM take 6 to 8 hours to return to normal behavior (Scanlan, Suchland et al. 2004). However, we observed that rats recovered as soon as the anesthesia wore off (~10 minutes).

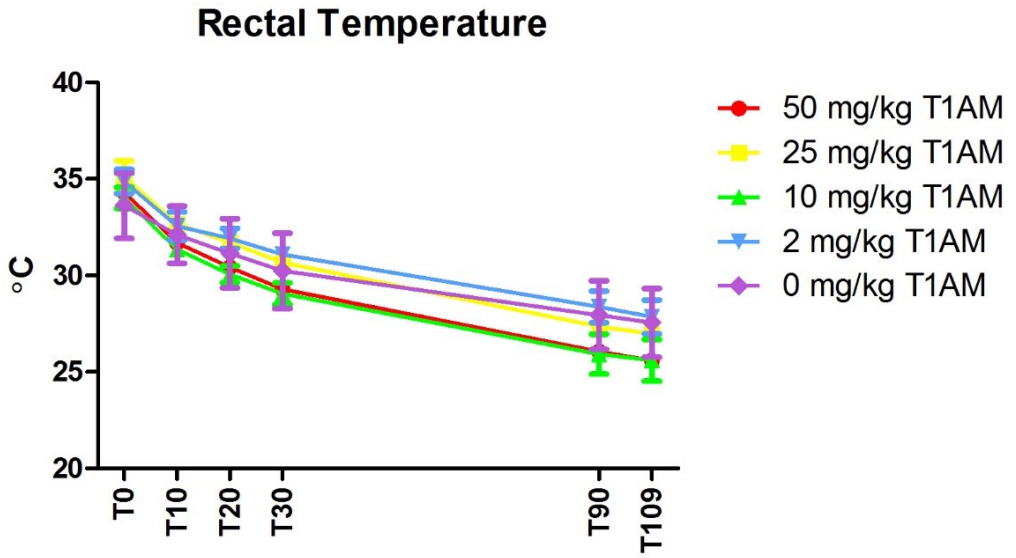


Figure 3.9. T1AM Pilot Study: Rectal temperature data.

Infusion of either 50 mg/kg T1AM (n=2), 25 mg/kg T1AM (n=2), 10 mg/kg T1AM (n=2), 2 mg/kg T1AM (n=2), or 0 mg/kg T1AM (n=2) was achieved by administering a single 1 ml/kg bolus. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Whole-Blood Parameters

If the mean survival was not different between hemorrhaged groups for a specific experiment, whole-blood parameters for that experiment will not be detailed.

Optimization of Composition

BHB Dose-Ranging Study

All treatment differences are summarized in Table 3.6 and Figure 3.10.

Acidosis is considered a good indicator of decreased oxygen delivery during the early stages of hemorrhagic shock. A pH of 7.30-7.35 is considered tolerable whereas a pH of ≤ 7.2 is considered life-threatening (Blalock 1940). Treatments differences in pH were observed at T0. This is more reflective of individual variation since all animals have been instrumented as per the protocol in the methods section and no infusion has taken place at this time point. All groups presented reductions in pH, this could be in part as a result in rectal temperature drops, even though results were temperature-corrected. The 4 M BHB and 2 M BHB treatments had lower pH than shams at T30 (p<0.05). However, the differences were only 0.04 and 0.15 points, respectively.

tHb decreases as a result of blood loss and fluid replacement (Gutierrez, Reines et al. 2004). Statistical differences in tHb were not observed until T30 between sham-operated animals and all hemorrhaged groups (p<0.0001) as the loss of blood is accompanied by a reduction in red cell mass (Carey, Lowery et al. 1971). A blood transfusion restored tHb levels close to baseline in hypovolemic animals.

sO_2 is a measure of oxygenation. It can be used to assess hypoxemia and imbalances in oxygen delivery (Cheatham, Block et al. 2008). sO_2 at T0 was slightly decreased in all groups, probably as a result of anesthesia as isoflurane has been known to cause dips in sO_2 at concentrations larger than 2% (Palahniuk and Shnider 1974).

pO_2 is an indicator of perfusion, or the oxygen delivery capacity of the lungs (Guyton and Hall 2001). pO_2 was higher in all hemorrhaged treatments compared to shams at T20 ($p<0.0001$), T30 ($p<0.01$), T30 ($p<0.0001$), T90 ($p<0.0001$), and T105 ($p<0.001$). This is contrary to the expectation that pO_2 will be depressed in shocked animals (Carey, Lowery et al. 1971). However, it reflects intact respiratory function.

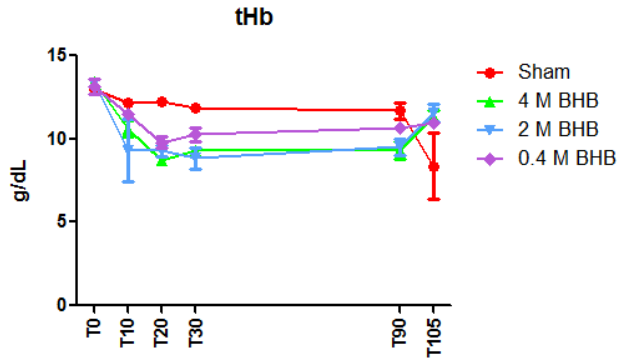
pCO_2 represents ventilation and denotes air exchange in the lungs and CO_2 elimination (Guyton and Hall 2001). Changes in pCO_2, though minimal and not statistically different between treatments ($p>0.05$), seemed to parallel changes in blood pressure.

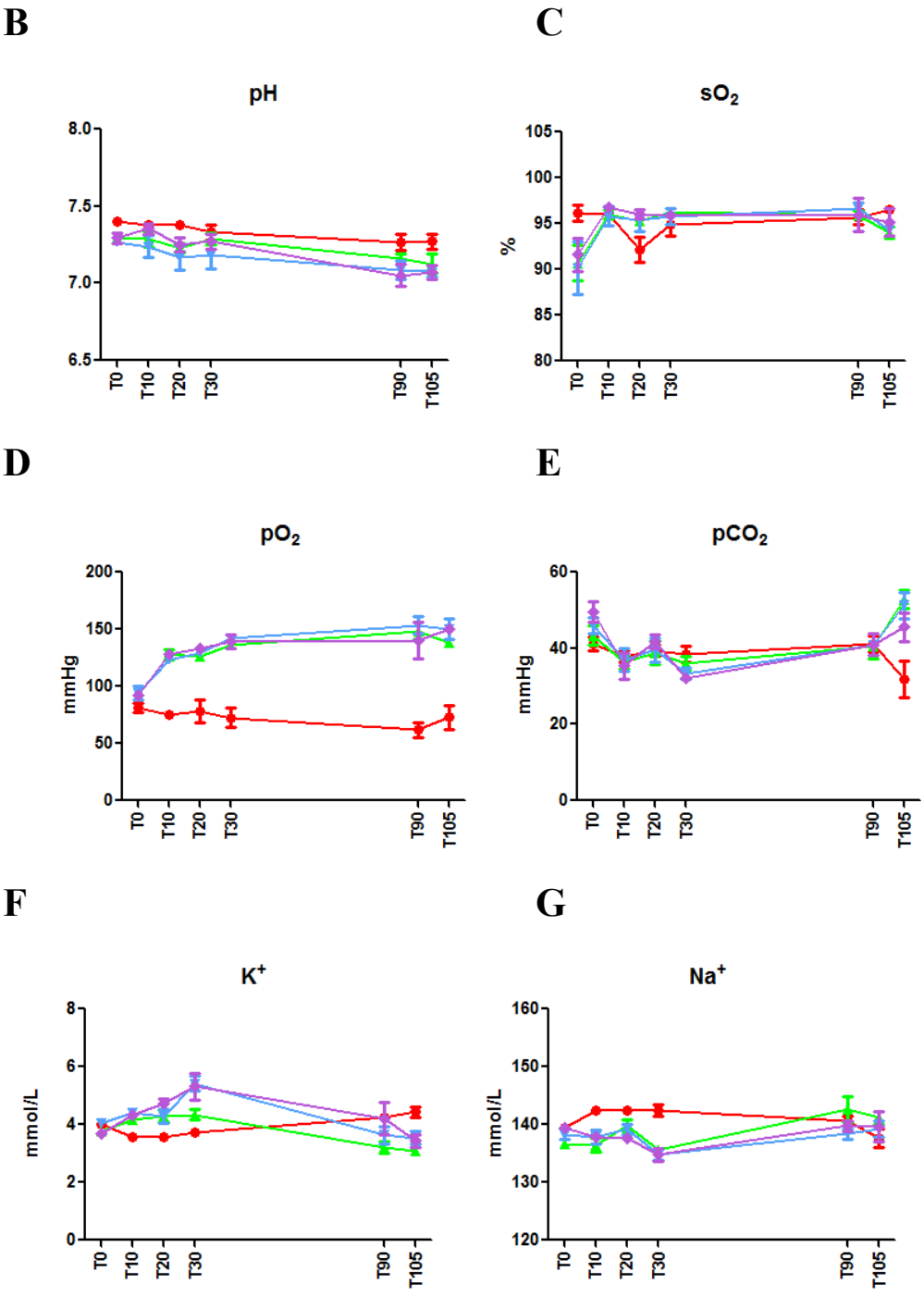
Hyperkalemia is expected to occur in sever shock. The Na-K pump requires ATP in order to function properly. As ATP levels decrease during ischemia, the cells lose their ability to maintain chemical differences between intracellular and extracellular fluids causing an increase in extracellular K^+ (Carey, Lowery et al. 1971). In rats subjected to hypovolemia, circulating K^+ levels increased as the hemorrhagic phase progressed but returned to levels close to baseline during the shock period. In contrast, blood K^+ in shams was lower from T0 to T10 but continually increased, resulting in statistically higher levels ($p<0.05$) when compared to all hemorrhaged groups at the end of the surgical procedure. However,

mean K^+ concentrations were not outside of the normal range (3.9-9.2 mmol/L) (Sharp and Villano 1998) at any time point in any group.

Na-K pump dysfunction also results in an increase of intracellular Na^+, therefore hemorrhage lowers blood Na^+ levels (Carey, Lowery et al. 1971). Since the BHB we used comes in its sodium salt form, circulating Na^+ increased upon infusion. The one-hour shock period allowed Na^+ to go back to homeostatic values. In shams, blood Na^+ increased after the first few blood samples and then slowly returned to its basal range.

A




B
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7.5
7.0
6.5
T0
T10
T20
T30
T90
T105
C
sO2
105
100
95
90
85
80
%
D
pO2
200
150
100
50
0
mmHg
E
pCO2
60
40
20
0
mmHg
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K+
8
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4
2
0
mmol/L
G
Na+
160
150
140
130
120
mmol/L

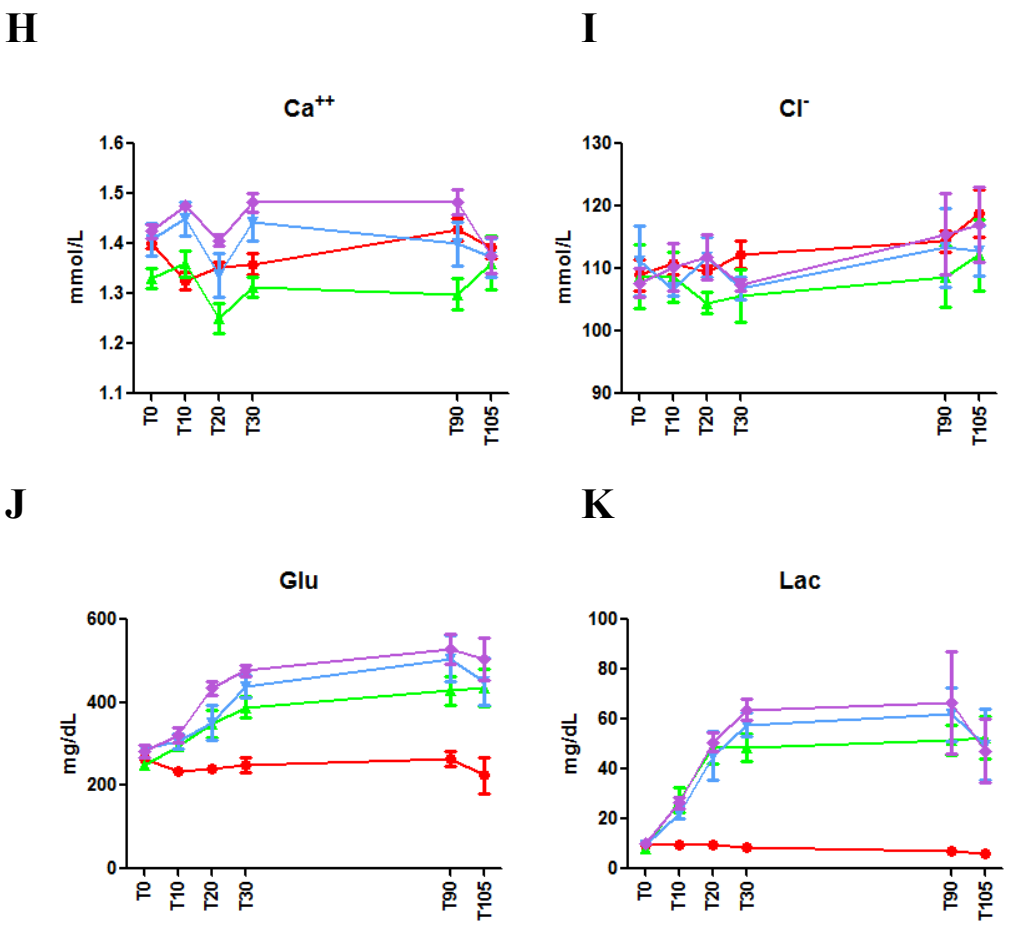


Figure 3.10. BHB Dose-Ranging Study: Whole-blood parameter data.

Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Table 3.6. BHB Dose-Ranging Study: Whole-blood parameter data.

					tHb (g/dL)							
					Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	13.02 ± 0.78	A	12.17 ± 0.95	A	12.24 ± 0.77	A	11.83 ± 0.86	A	11.69 ± 1.54	A	8.35 ± 5.28	A
4 M BHB	13.47 ± 0.45	A	10.65 ± 1.24	A	8.70 ± 0.20	BC	9.30 ± 0.36	B	9.30 ± 1.25	B	11.33 ± 0.97	A
2 M BHB	13.34 ± 0.60	A	9.30 ± 4.21	A	9.30 ± 0.75	BC	8.84 ± 1.41	B	9.50 ± 1.07	B	11.55 ± 1.02	A
0.4 M BHB	13.13 ± 1.09	A	11.52 ± 0.46	A	9.78 ± 0.86	B	10.26 ± 0.89	B	10.63 ± 0.32	AB	10.95 ± 0.21	A
					pH							
					Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	7.4 ± 0.04	A	7.38 ± 0.08	A	7.38 ± 0.06	A	7.33 ± 0.16	A	7.27 ± 0.19	A	7.27 ± 0.14	A
4 M BHB	7.30 ± 0.08	B	7.30 ± 0.13	A	7.23 ± 0.08	B	7.29 ± 0.10	A	7.16 ± 0.09	A	7.13 ± 0.16	A
2 M BHB	7.27 ± 0.07	B	7.24 ± 0.16	A	7.17 ± 0.19	B	7.18 ± 0.20	A	7.09 ± 0.14	A	7.08 ± 0.09	A
0.4 M BHB	7.29 ± 0.08	B	7.35 ± 0.08	A	7.25 ± 0.11	AB	7.27 ± 0.11	A	7.05 ± 0.15	A	7.07 ± 0.08	A
					sO_2 (%)							
					Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	96.15 ± 2.80	A	95.99 ± 1.46	A	92.14 ± 4.21	A	94.90 ± 4.04	A	95.66 ± 2.59	A	96.51 ± 0.70	A
4 M BHB	90.66 ± 5.10	A	96.01 ± 1.35	A	95.23 ± 1.16	A	96.28 ± 0.98	A	95.92 ± 1.21	A	94.02 ± 1.62	A
2 M BHB	90.12 ± 6.40	A	95.80 ± 2.17	A	95.38 ± 2.66	A	95.76 ± 1.94	A	96.60 ± 1.51	A	94.30 ± 0.41	A
0.4 M BHB	91.58 ± 4.34	A	96.72 ± 0.40	A	96.02 ± 1.29	A	95.88 ± 0.59	A	95.95 ± 2.62	A	95.20 ± 2.12	A
					pO_2 (mmHg)							
					Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	81.41 ± 10.83	A	75.62 ± 8.32	B	78.30 ± 13.86	B	72.30 ± 14.85	B	61.76 ± 15.28	B	72.73 ± 26.69	B
4 M BHB	92.3 ± 0.57	A	127.75 ± 8.18	A	126.50 ± 0.71	A	136.00 ± 5.29	A	148.50 ± 17.91	A	137.67 ± 4.97	A
2 M BHB	94.10 ± 14.54	A	123.00 ± 3.61	A	128.67 ± 5.35	A	142.00 ± 1.41	A	153.60 ± 17.26	A	150.20 ± 19.59	A
0.4 M BHB	92.56 ± 8.25	A	128.33 ± 8.12	A	132.75 ± 3.20	A	139.00 ± 8.49	A	140.45 ± 31.79	A	150.00 ± 6.25	A

pCO_2 (mmHg)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	41.1 ± 5.62	A	37.84 ± 4.73	A	39.37 ± 4.63	A	38.39 ± 6.86	A	41.18 ± 6.85	A	31.94 ± 13.56	A
4 M BHB	43.34 ± 6.98	A	36.38 ± 4.45	A	38.87 ± 8.00	A	36.01 ± 4.41	A	40.65 ± 8.18	A	52.93 ± 4.92	A
2 M BHB	45.83 ± 4.70	A	36.94 ± 6.66	A	39.55 ± 7.13	A	33.38 ± 3.24	A	40.95 ± 0.75	A	51.24 ± 6.10	A
0.4 M BHB	49.53 ± 6.75	A	35.32 ± 8.16	A	41.71 ± 3.88	A	32.23 ± 2.18	A	40.97 ± 3.97	A	45.55 ± 5.45	A
K^+ (mmol/L)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	4.02 ± 0.26	A	3.57 ± 0.33	B	3.56 ± 0.25	B	3.75 ± 0.42	B	4.26 ± 0.50	A	4.44 ± 0.52	A
4 M BHB	3.76 ± 0.37	A	4.17 ± 0.24	A	4.28 ± 0.50	A	4.34 ± 0.49	B	3.20 ± 0.53	B	3.10 ± 0.28	B
2 M BHB	4.05 ± 0.22	A	4.40 ± 0.12	A	4.30 ± 0.58	A	5.42 ± 0.58	A	3.64 ± 0.67	AB	3.54 ± 0.48	B
0.4 M BHB	3.70 ± 0.24	A	4.32 ± 0.32	A	4.73 ± 0.38	A	5.32 ± 1.02	A	4.20 ± 1.14	AB	3.43 ± 0.38	B
Na^+ (mmol/L)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	139.40 ± 1.65	A	142.36 ± 2.25	A	142.50 ± 1.96	A	142.36 ± 3.23	A	140.55 ± 2.95	A	137.63 ± 4.31	A
4 M BHB	136.71 ± 1.25	B	136.43 ± 3.05	B	139.83 ± 2.40	A	135.71 ± 1.11	BC	142.71 ± 5.82	A	141.17 ± 2.64	A
2 M BHB	138.33 ± 2.16	AB	137.80 ± 2.59	B	139.17 ± 2.14	A	134.80 ± 2.05	C	138.40 ± 2.07	A	139.20 ± 3.03	A
0.4 M BHB	139.50 ± 1.38	A	137.83 ± 1.33	B	137.67 ± 1.75	A	134.8 ± 2.39	C	139.75 ± 1.71	A	139.67 ± 4.51	A
Ca^{++} (mmol/L)												
	Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	1.40 ±0.03	AB	1.33 ± 0.06	C	1.36 ± 0.03	AB	1.36 ± 0.07	BC	1.43 ± 0.07	A	1.39 ± 0.06	A
4 M BHB	1.33 ± 0.05	B	1.36 ± 0.07	BC	1.25 ± 0.07	BC	1.31 ± 0.05	C	1.30 ± 0.08	B	1.36 ± 0.13	A
2 M BHB	1.41 ± 0.08	AB	1.45 ± 0.08	AB	1.34 ± 0.11	AB	1.4 ± 0.09	AB	1.40 ± 0.10	AB	1.37 ± 0.09	A
0.4 M BHB	1.43 ± 0.03	A	1.48 ± 0.02	A	1.41 ± 0.03	A	1.48 ± 0.04	A	1.48 ± 0.05	A	1.38 ± 0.06	A

	Cl^- (mmol/L) Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	109.00 ± 7.83	A	110.91 ± 6.04	A	109.50 ± 2.76	A	112.18 ± 7.29	A	114.36 ± 5.78	A	118.89 ± 10.62	A
4 MBHB	108.71 ± 13.49	A	108.57 ±10.57	A	104.50 ± 4.23	A	105.57 ± 11.15	A	108.71 ± 12.83	A	112.17 ± 13.83	A
2 MBHB	111.17 ± 13.79	A	106.60 ± 2.30	A	111.83 ± 8.06	A	106.80 ± 3.90	A	113.40 ± 14.15	A	112.80 ± 9.04	A
0.4 MBHB	107.67 ± 5.57	A	110.17 ± 9.30	A	111.83 ± 9.00	A	107.40 ± 2.07	A	115.50 ± 13.10	A	117.00 ± 10.39	A
	Glu (mg/dL) Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	263.10 ± 23.85	A	234.36 ± 32.41	B	240.20 ± 35.12	B	249.18 ± 56.80	B	264.46 ± 59.72	B	224.00 ± 123.63	B
4 MBHB	250.57 ± 30.20	A	294.43 ± 24.23	A	348.00 ± 78.50	A	388.86 ± 69.53	A	428.57 ± 92.99	A	434.83 ± 110.41	A
2 MBHB	289.33 ± 21.77	A	306.00 ± 41.73	A	350.33 ± 103.14	A	439.00 ± 59.55	A	505.60 ± 126.29	A	451.80 ± 127.70	A
0.4 MBHB	283.33 ± 36.43	A	320.67 ± 43.88	A	434.83 ± 39.09	A	476.20 ± 28.62	A	529.00 ± 72.80	A	504.67 ± 86.52	A
	Lac (mg/dL) Mean ± SD											
	T0		T10		T20		T30		T90		T105	
Sham	9.50 ± 4.06	A	9.70 ± 3.92	B	9.40 ± 3.20	B	8.82 ± 2.52	B	7.30 ± 1.26	B	5.86 ± 4.38	B
4 MBHB	8.00 ± 1.83	A	27.43 ± 13.24	A	48.67 ± 15.68	A	48.57 ± 14.14	A	51.57 ± 16.39	A	52.50 ± 21.04	A
2 MBHB	9.67 ± 4.46	A	22.20 ± 4.60	A	45.33 ± 23.30	A	57.80 ± 10.73	A	62.00 ± 24.11	A	49.80 ± 32.27	A
0.4 MBHB	10.33 ± 4.18	A	26.33 ± 5.92	A	50.67 ± 9.25	A	63.80 ± 9.86	A	66.75 ± 41.06	A	47.33 ± 22.50	AB

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: BHB-β-Hydroxybutyrate.

A slight hypocalcemia can be expected as a result of hemorrhagic shock and ischemia/reperfusion injury as there is a rapid intracellular Ca^{++} influx as there is a disruption in Ca++ channel activity (Carey, Lowery et al. 1971). Ca^{++} blood concentrations varied between groups enough to show statistical differences at T0 ($p<0.05$). However, this is just reflective of individual variation between the animals assigned to each group and the Ca^{++} levels were not outside of the reference values (1.2-1.6 mmol/L) (Sharp and Villano 1998). With blood withdrawal, circulating Ca^{++} increased but they decreased upon infusion. In fact, Ca^{++} levels somewhat mirrored the curves for Na^{+} and MAP. Nonetheless, these fluctuations remained within the normal value range.

Hypochloremia may occur in hemorrhagic shock as cellular membrane dysfuction results in an intracellular uptake of Cl^{-} (Carey, Lowery et al. 1971).Circulating Cl^{-} levels fluctuated without a specific pattern or trend but tended towards the higher end of the reference levels (84-110 mmol/L) (Sharp and Villano 1998).

Gluconeogeneiss is increased early during shock as a response to increased catecholamine (e.g. adrenalin) release. Also, the shift from aerobic to anaerobic metabolism results in an increase in lactate production (Carey, Lowery et al. 1971). Sham-operated animals had lower Glu and Lac levels than all other treatments

(p<0.05) at all time points except T0, consistent with a normal response to shock (Carey, Lowery et al. 1971).

Melatonin Dose-Ranging Study II

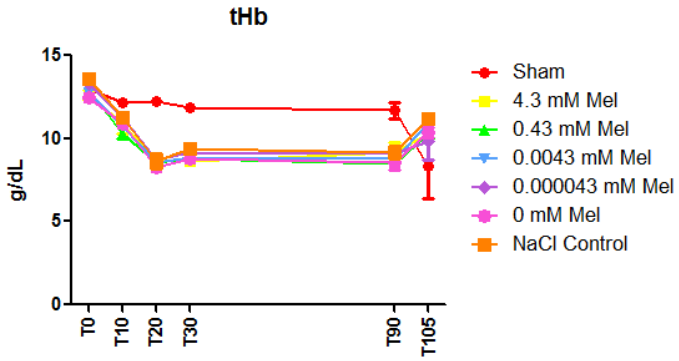
Differences between treatments are shown in Table 3.7 and Figure 3.11.

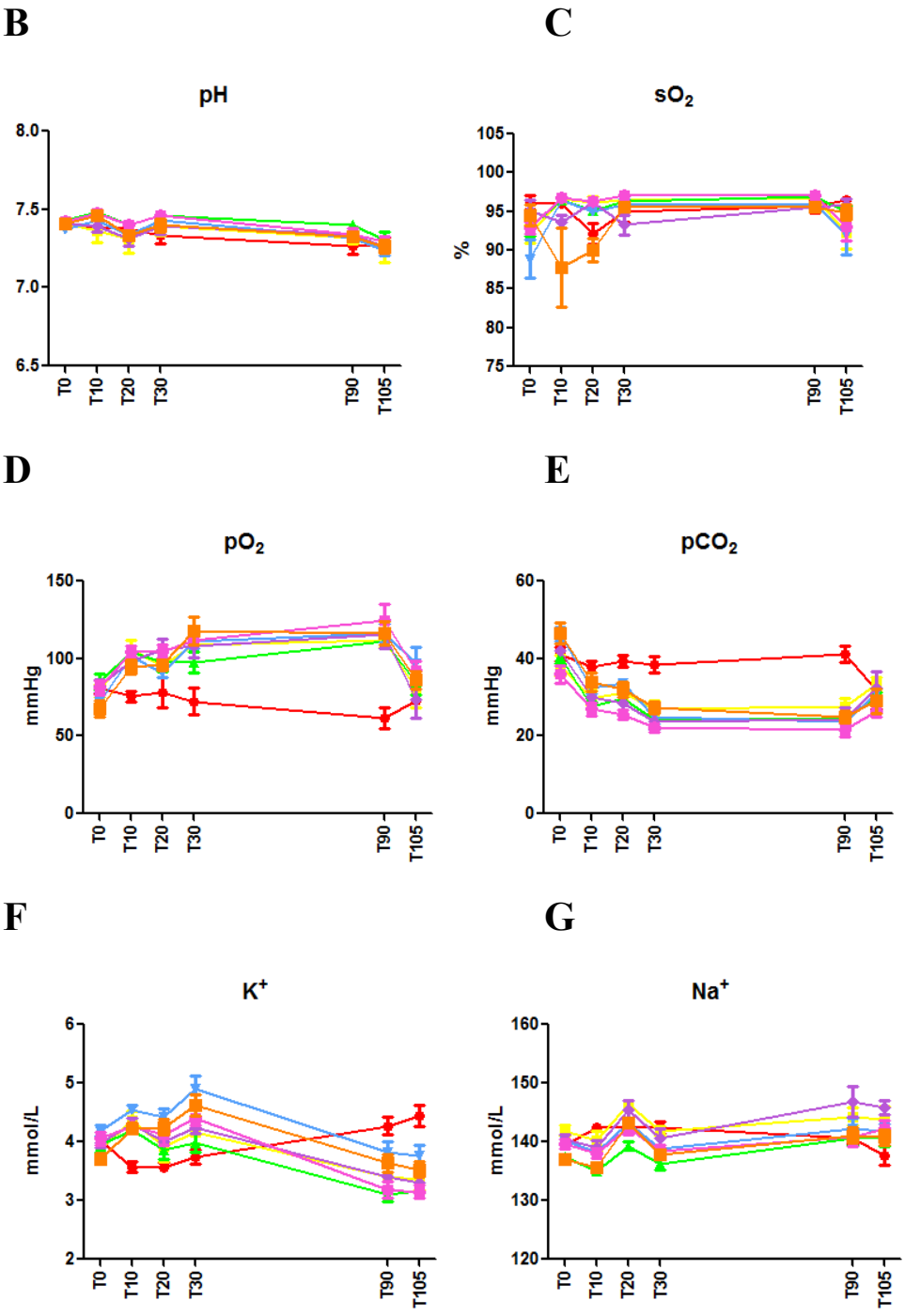
When it comes to tHb, all groups were indistinguishable at T0. tHb was consistently higher in shams compared to hemorrhaged animals. Those differences were no longer present after a blood transfusion.

pO_2 was consistently lower in shams compared to all other treatments except at T0 and T105. The opposite was observed with pCO_2.

K^+ levels in the hemorrhaged groups seemed to mirror MAP. In the sham group, circulating K^+ decreased initially and then gradually increased.

A




B
pH
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7.5
7.0
6.5
T0
T10
T20
T30
T90
T105
C
sO2
105
100
95
90
85
80
75
%
D
pO2
150
100
50
0
mmHg
E
pCO2
60
40
20
F
K+
6
5
4
3
2
mmol/L
G
Na+
160
150
140
130
120

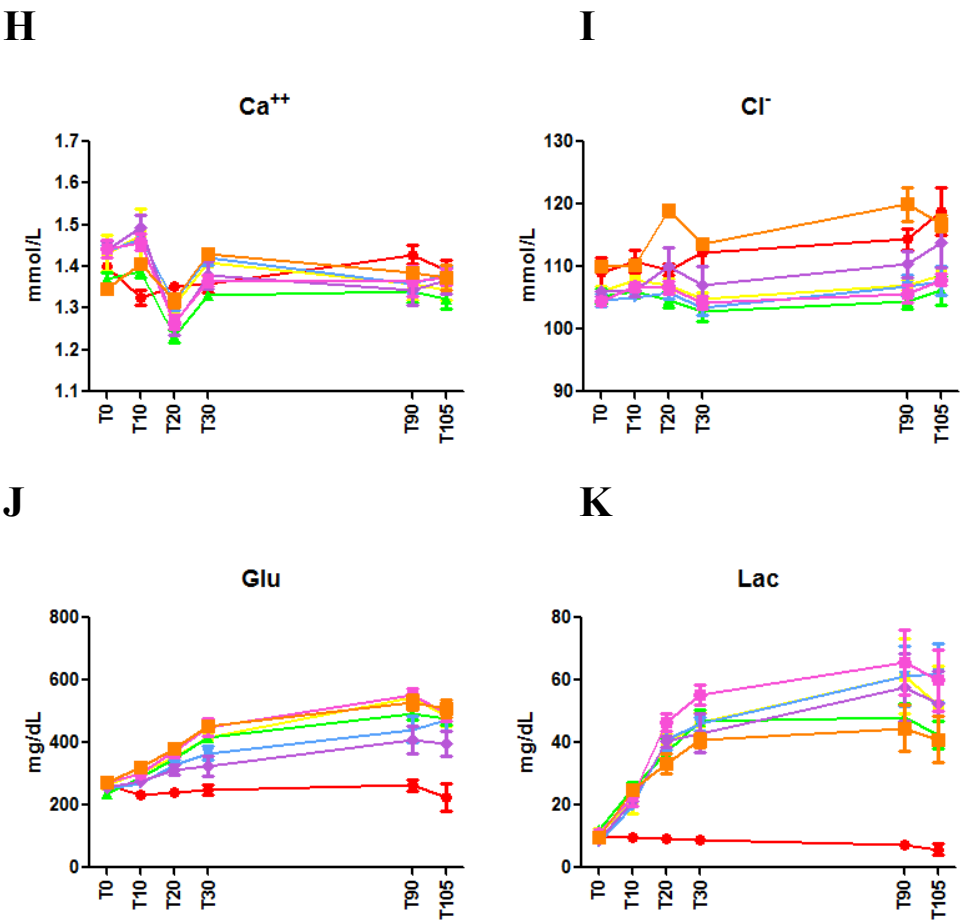

Figure 3.11. Melatonin Dose-Ranging Study II: Whole-blood parameter data.

Infusion of either 4.3 mM melatonin (n=10), 0.43 mM melatonin (n=10), 0.0043 mM melatonin (n=10), 0.000043 mM melatonin (n=10), or 0 mM melatonin (n=10) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=10). Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

In hemorrhaged animals, blood Na^+ decreased with blood withdrawals and increased with treatment infusion. Over the one-hour shock period, circulating Na^+ increased slightly. In sham animals, Na^+ levels stayed relatively constant.

Blood Ca^{++} concentrations varied and seemed to somewhat mirror MAP. However, those changes never fluctuated beyond the reference values for Ca^{++}.

Cl^- was higher in the animals infused with the NaCl control treatment from T20 onwards.

Table 3.7. Melatonin Dose-Ranging Study II: Whole-blood parameter data.

tHb (g/dL)												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	13.02 ± 0.78	A	12.17 ± 0.95	A	12.24 ± 0.77	A	11.83 ± 0.86	A	11.69 ± 1.54	A	8.35 ± 5.28	A
4.3 mM Mel	12.60 ± 0.92	A	10.91 ± 1.58	B	8.67 ± 0.76	B	8.61 ± 0.53	B	9.16 ± 1.55	B	10.30 ± 0.59	A
0.43 mM Mel	12.90 ± 0.80	A	10.30 ± 0.82	B	8.59 ± 0.51	B	8.76 ± 0.61	B	8.50 ± 0.63	B	10.31 ± 0.91	A
0.0043 mM Mel	12.74 ± 0.81	A	10.72 ± 0.75	B	8.55 ± 0.32	B	8.89 ± 0.46	B	8.78 ± 0.45	B	10.90 ± 0.84	A
0.000043 mM Mel	13.30 ± 0.65	A	11.29 ± 0.69	AB	8.73 ± 0.38	B	9.17 ± 0.51	B	9.11 ± 0.28	B	9.85 ± 2.26	A
0 mM Mel	12.49 ± 0.70	A	10.89 ± 0.44	B	8.28 ± 0.46	B	8.76 ± 0.33	B	8.53 ± 1.16	B	10.38 ± 0.92	A
NaCl Control	13.61 ± 0.59	A	11.29 ± 0.36	AB	8.63 ± 1.40	B	9.36 ± 0.40	B	9.16 ± 1.18	B	11.18 ± 0.56	A
pH												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	7.40 ± 0.04	A	7.38 ± 0.08	A	7.38 ± 0.06	A	7.33 ± 0.20	A	7.27 ± 0.19	A	7.27 ± 0.14	A
4.3 mM Mel	7.41 ± 0.06	A	7.37 ± 0.21	A	7.31 ± 0.22	A	7.39 ± 0.17	A	7.32 ± 0.11	A	7.23 ± 0.19	A
0.43 mM Mel	7.43 ± 0.03	A	7.49 ± 0.06	A	7.40 ± 0.08	A	7.46 ±0.06	A	7.40 ± 0.07	A	7.31 ± 0.15	A
0.0043 mM Mel	7.372 ± 0.05	A	7.43 ± 0.07	A	7.33 ± 0.09	A	7.43 ± 0.12	A	7.32 ± 0.08	A	7.24 ± 0.12	A
0.000043 mM Mel	7.41 ± 0.04	A	7.40 ± 0.12	A	7.31 ± 0.13	A	7.39 ± 0.14	A	7.34 ± 0.08	A	7.26 ± 0.06	A
0 mM Mel	7.42 ± 0.07	A	7.50 ± 0.05	A	7.40 ± 0.06	A	7.46 ± 0.04	A	7.35 ± 0.10	A	7.29 ± 0.10	A
NaCl Control	7.41 ± 0.05	A	7.46 ± 0.07	A	7.33 ± 0.09	A	7.40 ± 0.12	A	7.32 ± 0.09	A	7.26 ± 0.13	A
sO_2 (%)												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	96.15 ± 2.79	A	96.00 ± 1.46	A	92.14 ± 4.21	BC	94.90 ± 4.04	A	95.66 ± 2.59	A	96.51 ± 0.70	A
4.3 mM Mel	92.14 ± 3.53	AB	96.66 ± 0.91	A	96.23 ± 1.56	AB	96.53 ± 1.07	A	96.71 ± 0.96	A	92.33 ± 5.14	A
0.43 mM Mel	93.04 ± 3.60	AB	96.41 ± 1.05	A	95.19 ± 0.99	AB	96.36 ± 0.43	A	96.92 ± 1.08	A	95.00 ± 2.16	A
0.0043 mM Mel	88.86 ± 7.34	B	96.54 ± 0.74	A	94.94 ± 1.69	AB	95.87 ± 2.45	A	96.06 ± 0.84	A	92.05 ± 8.94	A
0.000043 mM Mel	95.19 ± 3.44	A	93.69 ± 2.06	A	96.24 ± 0.86	A	93.27 ± 3.47	A	95.53 ± 1.32	A	95.84 ± 1.83	A
0 mM Mel	92.88 ± 2.20	AB	96.81 ± 1.19	A	96.28 ± 1.20	A	97.06 ± 0.92	A	97.00 ± 1.25	A	93.07 ± 5.72	A
NaCl Control	94.47 ± 4.06	AB	87.82 ± 16.11	A	90.00 ± 4.81	C	95.56 ± 1.53	A	95.62 ± 1.80	A	94.87 ± 3.13	A

pO_2 (mmHg) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	81.41 ± 10.83	A	75.62 ± 8.32	B	78.30 ± 13.86	A	72.30 ± 14.85	B	61.76 ± 15.28	B	72.73 ± 26.69	A
4.3 mM Mel	79.36 ± 11.92	A	105.84 ± 12.83	A	95.62 ± 8.54	A	109.00 ± 3.27	A	112.03 ± 11.21	A	80.08 ± 27.00	A
0.43 mM Mel	85.03 ± 14.36	A	104.64 ± 6.71	A	97.85 ± 8.70	A	97.60 ± 6.73	AB	111.10 ± 7.30	A	84.61 ± 20.69	A
0.0043 mM Mel	72.02 ± 4.34	A	101.07 ± 6.83	A	90.88 ± 6.60	A	110.75 ± 10.62	A	116.33 ± 16.71	A	96.50 ± 33.40	A
0.000043 mM Mel	82.25 ± 9.68	A	98.07 ± 9.90	A	105.73 ± 14.34	A	107.90 ± 14.66	A	115.90 ± 21.23	A	73.70 ± 26.66	A
0 mM Mel	81.01 ± 12.77	A	104.70 ± 9.00	A	104.63 ± 11.33	A	111.60 ± 2.07	A	124.33 ± 26.65	A	92.29 ± 16.95	A
NaCl Control	67.78 ± 15.28	A	94.82 ± 13.21	A	95.78 ± 6.74	A	117.60 ± 21.84	A	116.20 ± 21.47	A	86.13 ± 15.78	A
pCO_2 (mmHg) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	41.10 ± 5.62	AB	37.84 ± 4.73	A	39.37 ± 4.63	A	38.39 ± 6.86	A	41.18 ± 6.85	A	31.94 ± 13.56	A
4.3 mM Mel	38.21 ± 7.90	AB	29.90 ± 4.73	B	31.25 ± 4.82	BC	26.94 ± 5.90	B	27.62 ± 6.62	B	33.29 ± 4.90	A
0.43 mM Mel	40.48 ± 4.64	AB	27.65 ± 3.31	B	29.74 ± 2.47	BC	24.43 ± 1.23	B	24.33 ± 2.32	B	29.84 ± 4.11	A
0.0043 mM Mel	46.21 ± 5.47	A	32.45 ± 5.12	AB	33.51 ± 3.75	AB	24.93 ± 5.70	B	23.89 ± 6.38	B	30.92 ± 4.98	A
0.000043 mM Mel	41.93 ± 3.29	AB	29.95 ± 2.50	B	28.56 ± 4.61	BC	23.84 ± 6.20	B	24.36 ± 8.48	B	32.56 ± 9.37	A
0 mM Mel	36.01 ± 7.15	B	26.95 ± 4.62	B	25.53 ± 4.15	C	22.27 ± 3.51	B	21.50 ± 4.69	B	26.34 ± 4.03	A
NaCl Control	46.55 ± 8.22	A	33.92 ± 7.90	AB	32.02 ± 5.92	B	27.31 ± 4.70	B	25.00 ± 4.24	B	29.04 ± 10.20	A
K^+ (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	4.02 ± 0.26	AB	3.57 ± 0.33	B	3.56 ± 0.25	B	3.75 ± 0.42	C	4.26 ± 0.50	A	4.44 ± 0.52	A
4.3 mM Mel	3.88 ± 0.31	AB	4.36 ± 0.44	A	3.92 ± 0.61	AB	4.16 ± 0.58	BC	3.40 ± 0.57	BC	3.37 ± 0.48	BC
0.43 mM Mel	3.93 ± 0.33	AB	4.20 ±.018	A	3.86 ± 0.45	AB	3.99 ± 0.45	BC	3.10 ± 0.32	C	3.16 ± 0.22	C
0.0043 mM Mel	4.19 ± 0.27	A	4.54 ± 0.25	A	4.43 ± 0.45	A	4.91 ± 0.69	A	3.82 ±0.57	AB	3.76 ± 0.58	B
0.000043 mM Mel	3.98 ± 0.23	AB	4.31 ± 0.28	A	4.00 ± 0.16	AB	4.25 ± 0.28	ABC	3.40 ± 0.64	BC	3.30 ±0.16	BC
0 mM Mel	4.06 ± 0.35	AB	4.26 ± 0.21	A	4.13 ± 0.62	AB	4.41 ± 0.38	ABC	3.19 ± 0.40	BC	3.15 ± 0.30	C
NaCl Control	3.71 ± 0.29	B	4.23 ± 0.29	A	4.25 ± 0.40	A	4.62 ± 0.56	AB	3.63 ± 0.42	ABC	3.53 ± 0.39	BC

Na^+ (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	139.40 ±1.65	ABC	142.36 ± 2.25	A	142.50 ±1.96	AB	142.36 ± 3.23	A	140.55 ± 2.95	A	137.63 ± 4.31	B
4.3 mM Mel	141.89 ± 2.62	A	140.14 ± 4.71	AB	146.50 ± 1.87	A	141.88 ± 2.30	AB	144.33 ± 4.50	A	143.86 ± 5.76	AB
0.43 mM Mel	137.44 ± 2.01	BC	135.00 ± 1.31	D	139.33 ± 2.35	B	136.25 ± 2.66	C	140.67 ± 3.84	A	140.67 ± 4.24	AB
0.0043 mM Mel	140.22 ± 1.79	AB	138.20 ± 1.69	BCD	143.20 ± 2.10	AB	138.82 ± 1.89	ABC	142.33 ± 5.00	A	141.82 ± 3.97	AB
0.000043 mM Mel	140.38 ± 2.39	AB	139.13 ± 3.23	ABC	145.38 ± 4.69	A	140.63 ± 4.44	AB	146.88 ± 7.30	A	145.80 ± 2.78	A
0 mM Mel	139.70 ± 2.54	ABC	138.00 ± 2.39	BCD	142.40 ± 3.78	AB	138.44 ± 2.79	BC	140.89 ± 4.76	A	142.33 ± 3.87	AB
NaCl Control	137.00 ± 1.33	C	135.70 ± 1.16	CD	143.30 ± 2.16	AB	137.78 ± 0.97	BC	141.11 ± 4.11	A	140.80 ± 4.19	AB
Ca^{++} (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	1.40 ± 0.03	AB	1.33 ± 0.06	B	1.35 ± 0.03	A	1.36 ±0.07	AB	1.43 ± 0.07	A	1.39 ± 0.06	A
4.3 mM Mel	1.43 ± 0.13	AB	1.47 ± 0.17	A	1.31 ± 0.05	ABC	1.41 ± 0.10	AB	1.36 ± 0.11	A	1.34 ± 0.07	A
0.43 mM Mel	1.37 ± 0.04	AB	1.39 ± 0.04	AB	1.23 ± 0.05	C	1.33 ± 0.02	B	1.34 ± 0.09	A	1.32 ± 0.07	A
0.0043 mM Mel	1.44 ± 0.05	A	1.47 ± 0.03	A	1.32 ± 0.07	AB	1.42 ± 0.06	A	1.36 ± 0.07	A	1.38 ± 0.05	A
0.000043 mM Mel	1.44 ± 0.06	A	1.49 ± 0.09	A	1.26 ± 0.07	BC	1.38 ± 0.10	AB	1.34 ± 0.10	A	1.37 ± 0.07	A
0 mM Mel	1.44 ± 0.06\	A	1.46 ± 0.06	A	1.27 ± 0.05	BC	1.37 ± 0.04	AB	1.36 ± 0.11	A	1.37 ± 0.06	A
NaCl Control	1.35 ± 0.03	B	1.41 ± 0.03	AB	1.32 ± 0.05	AB	1.43 ± 0.03	A	1.39 ± 0.04	A	1.37 ± 0.09	A
Cl^- (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	109 ± 7.83	AB	110.91 ± 6.04	A	109.50 ± 2.76	B	112.18 ± 7.29	A	114.36 ± 5.78	AB	118.88 ± 10.62	A
4.3 mM Mel	106.11 ± 2.37	AB	107.83 ± 0.75	ABC	107.00 ± 2.68	B	104.88 ± 2.90	B	107.11 ± 2.37	C	108.71 ± 1.80	ABC
0.43 mM Mel	105.22 ± 3.87	AB	106.13 ± 3.31	BC	104.71 ± 3.30	B	102.75 ± 4.06	B	104.43 ± 2.94	C	106.14 ± 6.10	C
0.0043 mM Mel	104.67 ± 3.00	AB	105.00 ± 1.94	C	105.80 ± 2.86	B	103.36 ± 3.56	B	106.89 ± 5.13	C	107.73 ± 7.46	C
0.000043 mM Mel	106.00 ± 2.20	AB	106.25 ± 2.87	ABC	110.00 ± 8.42	B	107.13 ± 8.48	AB	110.38 ± 5.78	BC	113.80 ± 8.90	ABC
0 mM Mel	104.50 ± 2.22	B	106.88 ± 2.23	ABC	106.70 ± 3.23	B	104.22 ± 2.82	B	105.63 ± 3.78	C	107.89 ± 2.76	BC
NaCl Control	110.10 ± 1.45	A	110.30 ± 1.16	AB	119.00 ± 2.91	A	113.70 ± 2.54	A	120.00 ± 8.02	A	116.89 ± 3.76	AB

Glu (mg/dL) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	263.10 ± 23.85	A	234.36 ± 32.41	C	240.20 ± 35.12	C	249.18 ± 56.80	D	264.46 ± 59.72	C	224.00 ± 123.63	B
4.3 mM Mel	266.45 ± 21.75	A	305.00 ± 29.16	AB	353.33 ± 25.89	AB	416.50 ± 28.54	ABC	544.38 ± 80.00	A	484.86 ± 82.33	A
0.43 mM Mel	237.56 ± 34.67	A	287.75 ± 40.62	AB	347.89 ± 40.76	AB	416.88 ± 37.50	ABC	491.45 ± 61.43	AB	478.50 ± 60.82	A
0.0043 mM Mel	254.56 ± 23.46	A	270.60 ± 33.59	BC	328.90 ± 53.78	AB	365.00 ± 73.13	BC	442.22 ± 125.97	AB	470.73 ± 115.67	A
0.000043 mM Mel	257.50 ± 22.37	A	276.25 ± 41.70	ABC	312.88 ± 46.91	B	324.13 ± 91.72	CD	408.63 ± 127.71	B	396.60 ± 86.07	A
0 mM Mel	270.80 ± 35.50	A	302.13 ± 27.12	AB	372.60 ± 57.96	AB	447.22 ± 83.94	AB	550.89 ± 63.18	A	493.13 ± 65.96	A
NaCl Control	274.10 ± 16.20	A	318.70 ± 15.83	A	381.30 ± 33.73	A	454.45 ± 34.45	A	529.56 ± 74.77	AB	506.70 ± 88.59	A
Lac (mg/dL) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	9.50 ± 4.06	A	9.70 ± 3.92	B	9.40 ± 3.20	C	8.82 ± 2.52	C	7.30 ± 1.25	B	5.86 ± 4.38	B
4.3 mM Mel	10.86 ± 4.71	A	21.60 ± 9.86	A	39.67 ± 8.12	AB	46.63 ± 8.07	AB	61.25 ± 34.02	A	52.14 ± 32.56	A
0.43 mM Mel	11.89 ± 3.95	A	25.38 ± 5.85	A	36.78 ± 13.77	AB	47.00 ± 9.20	AB	48.22 ± 12.40	A	42.50 ± 12.26	AB
0.0043 mM Mel	8.22 ± 2.28	A	19.50 ± 2.88	A	40.70 ± 9.37	AB	46.00 ± 7.36	AB	61.22 ± 28.94	A	62.18 ± 31.21	A
0.000043 mM Mel	8.88 ± 3.09	A	21.25 ± 3.81	A	40.38 ± 5.07	AB	43.00 ± 17.78	AB	57.75 ± 30.29	A	52.40 ± 23.05	A
0 mM Mel	10.70 ± 4.50	A	22.75 ± 8.65	A	46.50 ± 9.25	A	55.11 ± 9.60	A	65.78 ± 30.95	A	59.89 ± 29.17	A
NaCl Control	9.80 ± 2.44	A	24.80 ± 4.05	A	33.40 ± 10.12	B	40.67 ± 6.71	B	44.56 ± 22.09	A	41.00 ± 23.26	AB

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: Mel-Melatonin.

Both Glu and Lac remained at basal levels in sham-operated animals while they constantly increased in hemorrhaged rats.

Sham rats were not included when comparing animals that lived to 10 days after 60% blood loss and those that died. All sham-operated animals survived to 10 days since they were not hemorrhaged and including them in these analyses could misrepresent the data. A T0, sO_2 was higher in non-survivors ($p<0.05$). tHb was lower in 10-day survivors at T10 ($p<0.05$). Blood Lac was higher at T20 in animals that did not live to 10 days ($p<0.05$). At T90, 10-day survivors had higher pH ($p<0.01$) and pCO_2 ($p<0.0001$); non-survivors had higher sO_2 ($p<0.05$), K^+ ($p<0.01$), and Lac ($p<0.001$). At T105, pH was higher in rats that lived to 10 days ($p<0.01$); animals that died before the 10 day end point had higher circulating Lac ($p<0.05$).

Sham-operated animals were also excluded from regression analyses. At T0, higher sO_2 ($p<0.05$; HR=1.22) resulted in lower survival. At T10, increased pH ($p<0.05$; HR=301.33) and increased tHb ($p<0.05$; HR=4.56) decreased survival. Reduced tHb at T20 ($p<0.05$; HR=0.16) reduced survival. Lower pCO_2 ($p<0.001$; HR=0.85) and higher K^+ ($p<0.01$) at T90 caused a reduction in survival. At T105, lower pH ($p<0.001$; HR=0.001) and higher Lac ($p<0.001$; HR=1.03) resulted in lower survival.

Histopathological Scoring

Histopathological analyses are important for our research because in trauma, death is mostly encountered at three points: 1) within the first hour, 2) within the next 24 hours, 3) after days or weeks (Baue, Faist et al. 2000). Since our post-operatory monitoring is not comprised of weeks, observing micro anatomical changes in different tissues provide information regarding the health status of the experimental subjects that survived the entire 10 days. It also helped us identify whether those animals would have kept living indefinitely or would have been likely to suffer health consequences in the near future. Histological scoring was only conducted on the second melatonin dose-ranging study.

A summary of the results obtained for brain, lung, and small intestine can be found in Figure 3.12. 10-day survivors administered 0.000043 mM melatonin had lower histopathological scores for intestine compared to those infused with 4.3 mM melatonin ($p<0.05$). However, the average score for this treatment represented only moderate damage, suggesting that, even though there was a stronger inflammatory response in this group, by 10 days after 60% blood loss the inflammation is being resolved.

Plasma Parameters

Blood samples were collected at six time points: 1) before hypotension (T0), 2) after 40% blood loss (T10), 3) 10 minutes after 40% blood loss (T20), 4) after

60% blood loss (T30), 5) one hour after 60% blood loss (T90), and 6) after blood return (T105). Since rats were anticoagulated with 10 IU of heparin via the left femoral vein, the centrifugation of the blood samples resulted in the separation of cells at the bottom and plasma, not serum, at the top.

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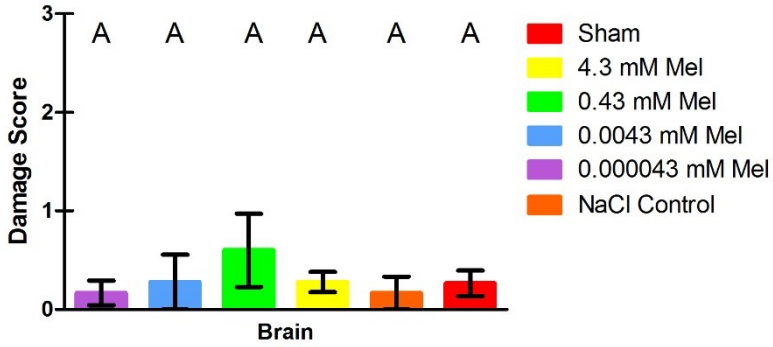

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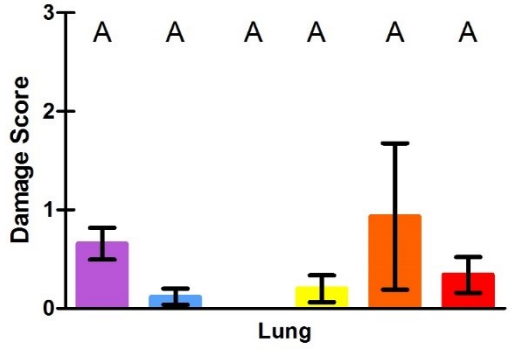

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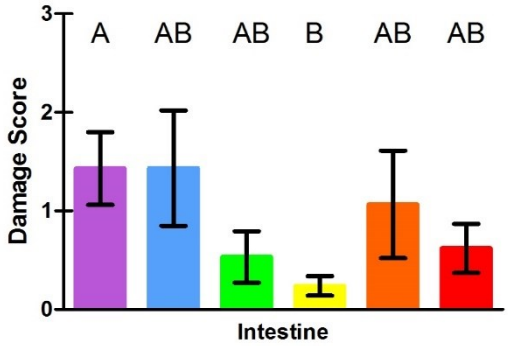

Figure 3.12. Melatonin Dose-Ranging Study II: Histopathological scores for (A) brain, (B) lung, and (C) small intestine.

Rats that survived to the experimental time point of 10 days were euthanized. Infusion of either 4.3 mM melatonin (n=7), 0.43 mM melatonin (n=7), 0.0043 mM melatonin (n=6), or 0.000043 mM melatonin (n=7) was achieved by administering a single 1 ml/kg bolus. The samples for the 0 mM melatonin treatment (n=3) were not viable for histological examination. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=3). All sham-operated animals (n=10) were included. Bars not present represent damage score values of 0. Data are depicted as mean ± SEM. Statistical significance is denoted by the presence of different letters.

BHB Dose-Ranging Study

Plasma BHB levels were higher in animals infused with 4 M BHB compared to those administered 0.4 M BHB at T20 ($p<0.05$) and T30 ($p<0.01$) (Figure 13; Table 3.8). These differences were expected because they were consistent with the different concentrations infused. At T105, the concentration of BHB in the plasma was higher in animals that died between day 0 and day 9 compared to those that survived until the 10 day end point ($p<0.05$). Lower BHB

levels at T20 (p<0.05) and circulating BHB at T105 (p<0.05) resulted in decreased survival.

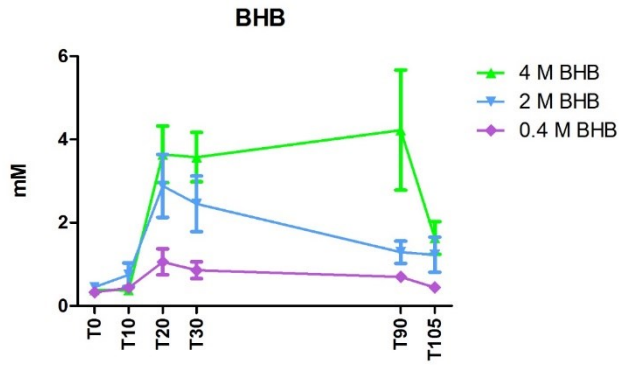

Figure 3.13. BHB Dose-Ranging Study: Plasma BHB levels.

Infusion of either 4 M BHB (n=6), 2 M BHB (n=5), or 0.4 M BHB (n=5) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4.3 mM melatonin and 10% DMSO. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Melatonin Dose-Ranging Study II

TNF-α was selected as a pro-inflammatory cytokine to assess inflammation. Since there were sample volume concerns, only one cytokine could be assessed. We chose TNF-α for two reasons: 1) It has been reported that maximum changes in TNF-α levels occur within the first few hours of hemorrhagic shock (Pati, Gerber et al. 2011), which coincides with the plasma samples we had available; and 2) TNF-α has been reported to differ from survivors and non survivors both in rat (Pati, Gerber et al. 2011) and human (Roumen, Hendriks et al. 1993) studies.

Table 3.8. BHB Dose-Ranging Study: Plasma BHB levels.

	BHB (mM) Mean ± SD											
	T0		T10		T20		T30		T90		T105	
4 MBHB	0.38 ± 0.20	A	0.38 ± 0.12	A	3.64 ± 1.93	A	3.57 ± 1.68	A	4.22 ± 4.08	A	1.63 ± 1.11	A
2 MBHB	0.45 ± 0.22	A	0.75 ± 0.75	A	2.88 ± 2.00	AB	2.45 ± 1.77	AB	1.29 ± 0.71	A	1.23 ± 1.21	A
0.4 MBHB	0.33 ± 0.12	A	0.43 ± 0.12	A	1.06 ± 0.77	B	0.86 ± 0.50	B	0.70 ± 0.21	A	0.44 ± 0.04	A

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: BHB-β-Hydroxybutyrate.

Circulating TNF-α levels were determined for a representative sample (n=3 per treatment) of animals from the sham group as well as animals administered 4.3 mM melatonin, 0.000043 mM melatonin, 0 mM melatonin, and the NaCl control for T20, T30, and T90. No differences were observed between any treatments at any time point (Figure 3.14; Table 3.9). However, when shams and NaCl controls were not considered, a dose dependent trend was observed at T30 where the higher the melatonin administered, the lower the circulating TNF-α. Also at T30, TNF-α plasma levels were lower in animals that lived until the end of the experiment compared to those that died prematurely ($p<0.05$). TNF-α did not seem to influence survival.

Table 3.9. Melatonin Dose-Ranging Study II: Plasma TNF-α levels.

TNF-α (pg/mL)						
	Mean ± SD					
	T20		T30		T90	
Sham	34.74 ± 3.12	A	10.73 ± 3.83	A	75.96 ± 48.09	A
4.3 mM Mel	23.55 ± 22.95	A	31.59 ± 45.72	A	20.34 ± 15.43	A
0.000043 mM Mel	19.74 ± 18.36	A	7.64 ± 9.01	A	41.33 ± 17.31	A
0 mM Mel	16.88 ± 10.52	A	38.26 ± 23.69	A	51.68 ± 28.49	A
NaCl Control	11.26 ± 14.33	A	18.05 ± 17.38	A	53.08 ± 22.92	A

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: Mel-Melatonin. TNF-α-Tumor Necrosis Factor Alpha.

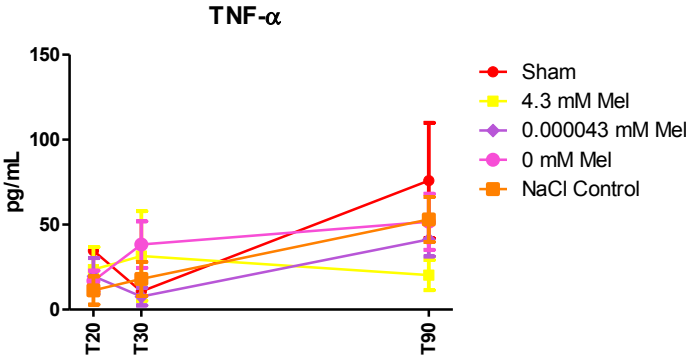

Figure 3.14. Melatonin Dose-Ranging Study II: Plasma TNF-α levels.

Infusion of either 4.3 mM melatonin (n=3), 0.000043 mM melatonin (n=3), or 0 mM melatonin (n=3) was achieved by administering a single 1 ml/kg bolus. All solutions contained 4 M BHB and 2% DMSO. A control group was also included and they were administered 4 M NaCl with 0.000043 mM melatonin in 2% DMSO (n=3). Sham-operated animals (n=3) were also included. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

CHAPTER IV: ISOTONIC EXPERIMENTS

Survival

Three-Hour Shock

The three hour shock protocol was conducted first as a pilot. The idea behind it was to see how far we could push the animals to failure and still resuscitate them successfully. *p*-values for all pairwise comparisons for 24 hours and 10 days are summarized in Table 4.1. No sham-operated animals were included in this study. The only statistical difference was observed at both 24 hours (Figure 4.1A) and 10 days (Figure 4.1B) between LR and 140 mM BHB with 4.3 mM melatonin ($p<0.05$). However, it is worth mentioning that only 3 out of the 20 individuals in the whole experiment survived to 10 days; the majority of deaths in all treatment groups occurred before 24 hours. Since the overall survival to 10 days of animals subjected to 60% blood loss and infused with isotonic fluids at a rate of one time the volume lost per hour for three hours is 15%, it becomes evident that the insult of 60% blood loss combined with 3 hours without a blood transfusion is too great for any isotonic resuscitation fluid to counteract. For these reasons, this study was left as a pilot with a sample size of 5 per treatment.

Table 4.1. Three-Hour Shock Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days.

Treatment		Mean ± SEM	
		24 Hrs	10 Days
LR		16.80 ± 5.58	4.50 ± 2.48
LR/4.3 mM Mel		12.13 ± 5.41	0.91 ± 0.55
140 mM BHB/1.5x10^{-6} mM Mel		9.60 ± 3.72	2.20 ± 2.01
140 mM BHB/4.3 mM Mel		5.89 ± 0.11	0.25 ± 0.01
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
LR/4.3 mM Mel	LR	0.4190	0.2360
140 mM BHB/1.5x10^{-6} mM Mel	LR	0.2210	0.2810
140 mM BHB/4.3 mM Mel	LR	0.0422	0.0422
140 mM BHB/1.5x10^{-6} mM Mel	LR/4.3 mM Mel	0.9050	0.9070
140 mM BHB/4.3 mM Mel	LR/4.3 mM Mel	0.4110	0.4110
140 mM BHB/4.3 mM Mel	140 mM BHB/1.5x10^{-6} mM Mel	0.1800	0.1800

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at 10 days. n=5 for all treatments. Statistically significant *p*-values are colored in red and underlined. Abbreviations: BHB-β-Hydroxybutyrate. LR-Lactated Ringer's. Mel-melatonin.

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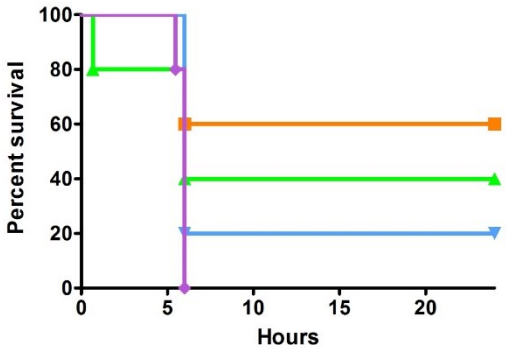

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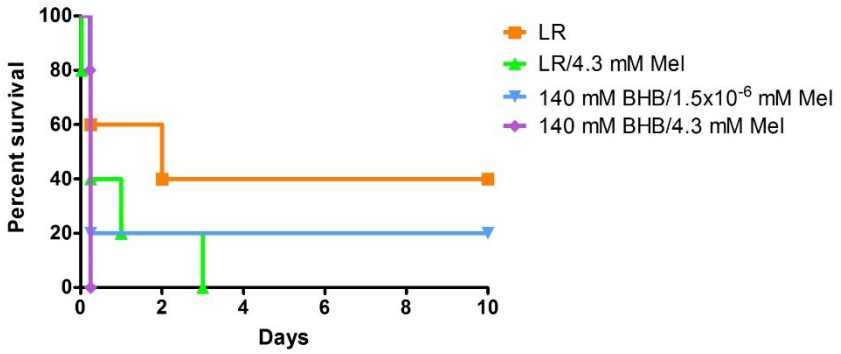

Figure 4.1. Three-Hour Shock: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days.

Infusion of either LR (n=5), LR plus 4.3 mM Mel (n=5), 140 mM BHB with 1.5x10-6 mM Mel (n=5) or 140 mM BHB with 4.3 mM Mel (n=5) was achieved by administering one time the volume of blood lost per hour for three hours. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss.

One-Hour Shock

A summary of all pairwise comparisons for both 24 hours and 10 days can be found in Table 4.2. The only statistical difference observed at 24 hours (Figure 4.2A) ($p<0.05$) was between sham-operated animals and those infused with 140 mM BHB with $1.5x10^{-6}$ mM melatonin. At 10 days (Figure 4.2B), the only group that was not statistically different from shams was the one administered LR ($p>0.05$). The 140 mM BHB with $1.5x10^{-6}$ mM melatonin treatment also had less successful survival times than LR and LR plus 4.3 mM melatonin ($p<0.05$). The addition of 4.3 mM melatonin to LR did not improve survival compared to LR on its own.

Since the three-hour shock experiments were left as a pilot study, only the one-hour shock will be described further.

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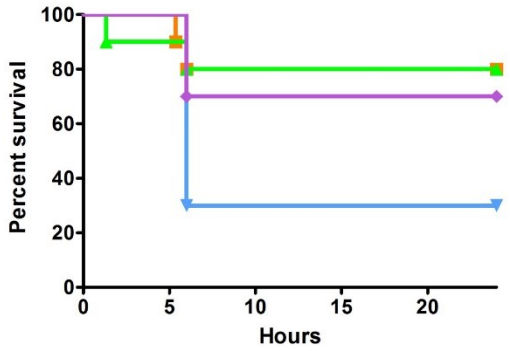

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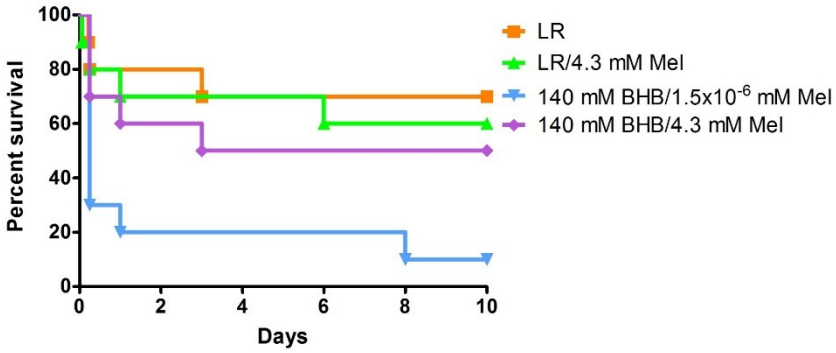

Figure 4.2. One-Hour Shock: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 24 hours and (B) 10 days.

Infusion of either LR (n=10), LR plus 4.3 mM Mel (n=10), 140 mM BHB with 1.5x10-6 mM Mel (n=10) or 140 mM BHB with 4.3 mM Mel (n=10) was achieved by administering three times the volume of blood lost per hour for one hour. Times on the x-axis reflect either hours (A) or days (B) after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

Table 4.2. One-Hour Shock Study: Mean survival time in animals subjected to 60% blood loss at 24 hours and 10 days.

Treatment		Mean ± SEM	
		24 Hrs	10 Days
LR		20.34 ± 3.28	7.35 ± 1.59
LR/4.3 mM Mel		19.93 ± 3.67	6.73 ± 1.57
140 mM BHB/1.5x10^{-6} mM Mel		11.40 ± 2.82	2.08 ± 1.18
140 mM BHB/4.3 mM Mel		18.60 ± 3.20	5.48 ± 1.62
Sham		24.00	10.00
Treatment Comparisons		*p*-value	
		24 Hrs	10 Days
LR	Sham	0.1280	0.0555
LR/4.3 mM Mel	Sham	0.1280	0.0229
140 mM BHB/1.5x10^{-6} mM Mel	Sham	0.0009	0.00005
140 mM BHB/4.3 mM Mel	Sham	0.0555	0.0087
LR/4.3 mM Mel	LR	0.9570	0.6910
140 mM BHB/1.5x10^{-6} mM Mel	LR	0.0643	0.0216
140 mM BHB/4.3 mM Mel	LR	0.7260	0.4660
140 mM BHB/1.5x10^{-6} mM Mel	LR/4.3 mM Mel	0.0643	0.0351
140 mM BHB/4.3 mM Mel	LR/4.3 mM Mel	0.7260	0.6780
140 mM BHB/4.3 mM Mel	140 mM BHB/1.5x10^{-6} mM Mel	0.0812	0.0521

Mean survival time calculated as the area under the Kaplan-Meier curve. Units are hours for the calculations at 24 hours and days for the calculations at 10 days. n=10 for all treatments. Statistically significant *p*-values are colored in red and underlined. Abbreviations: BHB-β-Hydroxybutyrate. LR-Lactated Ringer's. Mel-melatonin.

Non-Inferiority Test

Non-inferiority analyses were conducted to establish if any of the isotonic resuscitation fluids employed in these experiments were non-inferior to the published hypertonic BHB/M. We declared non-inferiority if the mean survival for each treatment was within a two-day Δ of the mean survival for the published hypertonic BHB/M. Only the infusion of LR, with or without melatonin, resulted in survival times within the specified boundary; both isotonic BHB/M formulations were declared inferior to the published BHB/M (Figure 4.3).

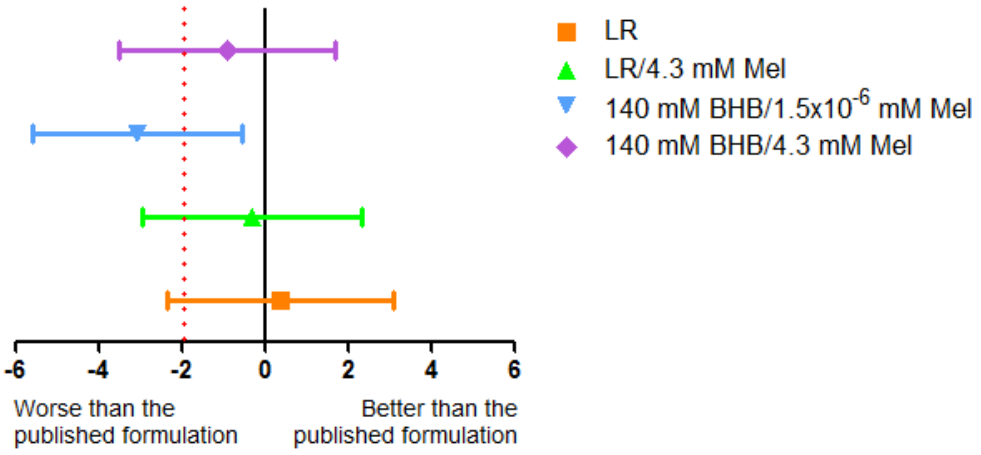

Figure 4.3 One-Hour Shock Study: Non-inferiority plot of animals subjected to 60% blood loss at 10 days.

Infusion of either LR (n=10), LR plus 4.3 mM Mel (n=10), 140 mM BHB with 1.5x10-6 mM Mel (n=10) or 140 mM BHB with 4.3 mM Mel (n=10) was achieved by administering three times the volume of blood lost per hour for one hour. Times on the x-axis reflect differences in survival times in days after achieving 60% blood loss. Data points are expressed as mean survival in days ± 95% Confidence Interval.

Physiological Constants

Treatment differences can be observed in Table 4.3 and Figure 4.4.

MAP was higher in shams compared to all hemorrhaged groups from T10 to T90 ($p<0.05$). The only differences ($p<0.05$) observed between the groups subjected to 60% blood loss were at T90 and T105 when the LR plus 4.3 mM melatonin group had lower MAP than all other treatment groups.

Sham-operated animals maintained higher HR than all hemorrhaged animals from T10 to T105 ($p<0.0001$). Rectal temperature was also consistently higher in shams compared to hemorrhaged animals from T0 to T105 ($p<0.0001$).

Table 4.3. One-Hour Shock Study: Physiological constant data.

MAP (mmHg)												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	99.20 ± 12.00	A	84.77 ± 12.57	A	84.77 ± 12.57	A	84.29 ± 11.70	A	97.45 ± 13.33	A	96.18 ± 12.66	A
LR	93.51 ± 10.41	A	26.02 ± 2.16	B	31.71 ± 6.56	B	20.13 ± 2.87		81.48 ± 11.33	B	102.34 ± 17.78	A
LR/4.3 mM Mel	89.31 ± 8.55	A	25.24 ± 1.22	B	30.49 ± 3.26	B	18.68 ± 2.47		56.20 ± 6.93	C	71.56 ± 10.00	B
140 mM BHB/1.5x10-6 mM Mel	87.82 ± 7.77	A	25.27 ± 1.10	B	33.88 ± 7.09	B	21.53 ± 2.72		75.11 ± 13.74	B	92.34 ± 10.35	A
140 mM BHB/4.3 mM Mel	92.41 ± 9.15	A	25.85 ± 1.26	B	31.90 ± 2.12	B	20.97 ± 3.07		72.73 ± 13.25	B	93.11 ± 17.21	A
HR (BPM)												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	360.32 ± 42.95	A	341.18 ± 56.17	A	333.03 ± 51.95	A	316.69 ± 55.00	A	299.54 ± 20.15	A	291.76 ± 23.90	A
LR	346.08 ± 47.54	A	206.47 ± 33.76	B	189.07 ± 20.01	B	266.35 ± 54.69	A	240.36 ± 28.02	B	223.33 ± 25.32	B
LR/4.3 mM Mel	335.15 ± 27.86	A	221.08 ± 50.56	B	201.34 ± 26.22	B	295.52 ± 48.44	A	219.39 ± 17.78	B	210.10 ± 17.37	B
140 mM BHB/1.5x10-6 mM Mel	329.40 ± 41.71	A	223.78 ± 33.76	B	202.17 ± 23.29	B	256.12 ± 50.43	A	239.59 ± 23.17	B	230.54 ± 23.72	B
140 mM BHB/4.3 mM Mel	338.71 ± 54.70	A	253.12 ± 49.37	B	216.29 ± 38.65	B	293.17 ± 66.03	A	237.58 ± 37.04	B	231.79 ± 31.77	B
Rectal Temperature (°C)												
Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	35.52 ± 0.37	A	33.83 ±0.48	A	32.86 ± 0.67	A	32.06 ± 0.65	A	29.35 ± 0.56	A	29.03 ± 0.54	A
LR	33.87 ± 0.60	B	31.01 ± 0.78	B	28.62 ± 0.87	B	27.21 ± 1.38	B	24.59 ± 1.41	B	24.33 ± 1.47	B
LR/4.3 mM Mel	33.78 ± .088	B	31.03 ± 0.95	B	28.68 ± 0.83	B	27.07 ± 0.79	B	25.16 ± 0.98	B	24.86 ± 0.92	B
140 mM BHB/1.5x10-6 mM Mel	33.87 ± 0.80	B	30.79 ± 0.92	B	28.46 ± 1.03	B	27.43 ± 1.00	B	25.23 ± 1.22	B	24.91 ± 1.21	B
140 mM BHB/4.3 mM Mel	33.93 ± 0.77	B	31.07 ± 0.82	B	28.60 ± 0.85	B	27.25 ± 0.87	B	25.37 ± 1.04	B	25.32 ± 0.97	B

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: BHB-β-Hydroxybutyrate. LR-Lactated Ringer's. Mel-Melatonin.

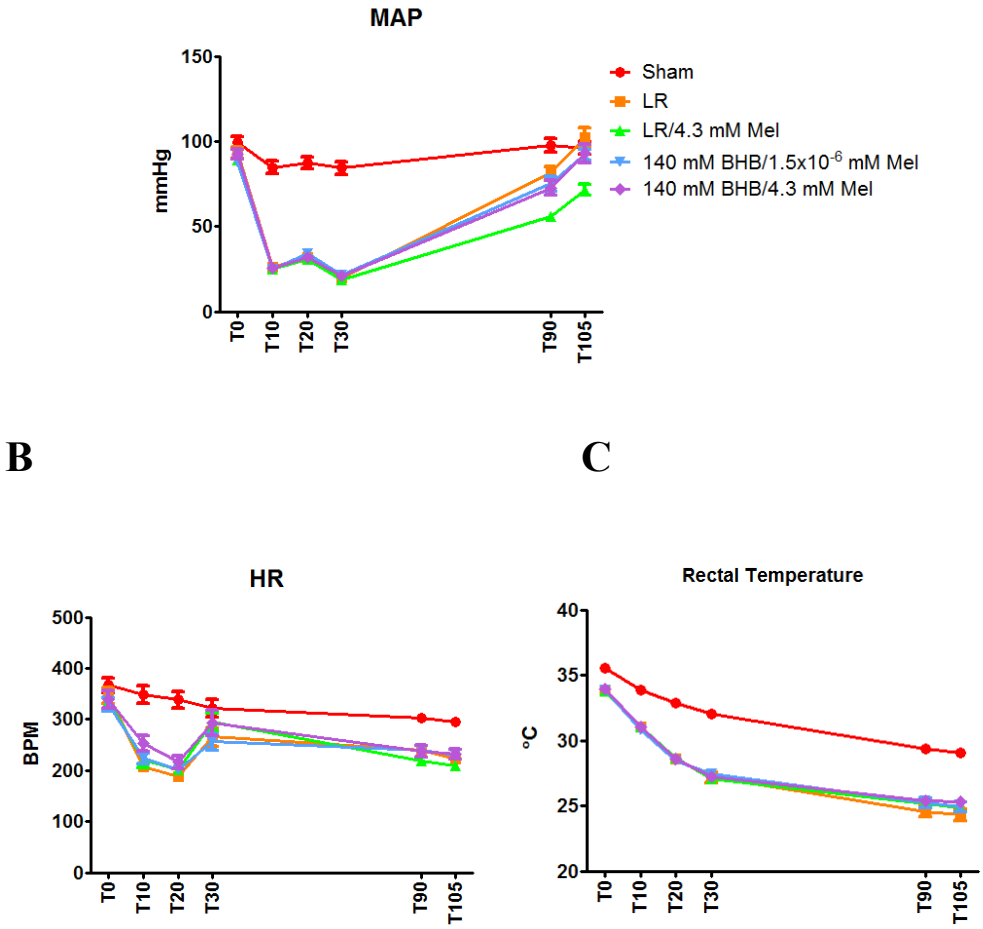

Figure 4.4. One-Hour Shock Study: Physiological constant data.

Infusion of either LR (n=10), LR plus 4.3 mM Mel (n=10), 140 mM BHB with 1.5x10-6 mM Mel (n=10) or 140 mM BHB with 4.3 mM Mel (n=10) was achieved by administering three times the volume of blood lost per hour for one hour. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Shams were not included in the comparisons between rats that survived to 10 days after 60% blood loss and those that did not. In sham operations the only

bleeding occurs for sampling and all the animals in the group lived to 10 days. Including them in these analyses could misstate the results. MAP was higher at T0 in animals that lived compared to those who died (p<0.05); the opposite was true at T20 (p<0.05). No other physiological parameters showed statistical differences between 10-day survivors and non-survivors.

Sham rats were also excluded from our Cox Proportional Hazards regression analyses. Higher MAP at T20 resulted in lower survival (p<0.05; HR=1.11). Lower HR was associated with lower survival at T0 (p<0.05; HR=0.98) and T30 (p<0.05; HR=0.99); the opposite was observed at T20 (p<0.01; HR=1.04). Rectal temperature had no effect on survival at any time point.

Whole Blood Parameters

All treatment differences are summarized in Table 4.4 and Figure 4.5.

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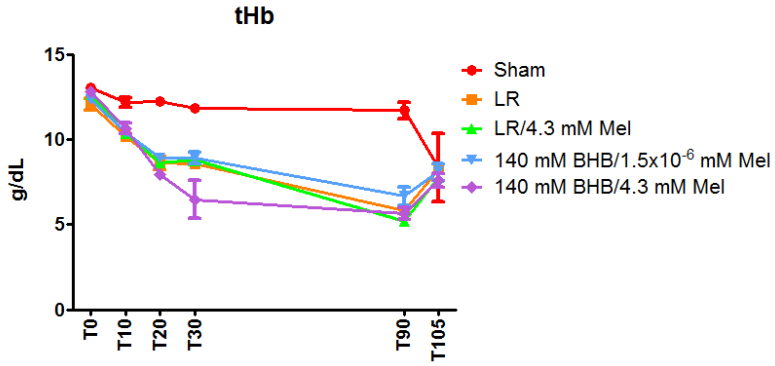

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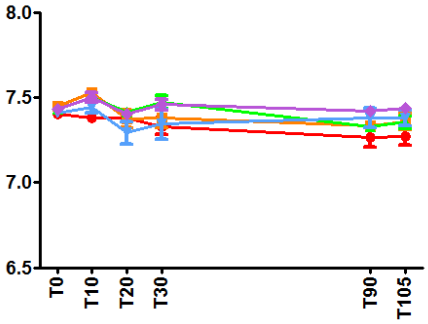
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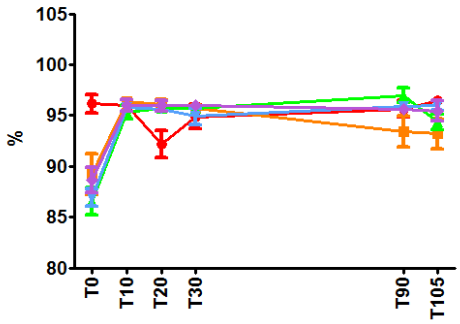
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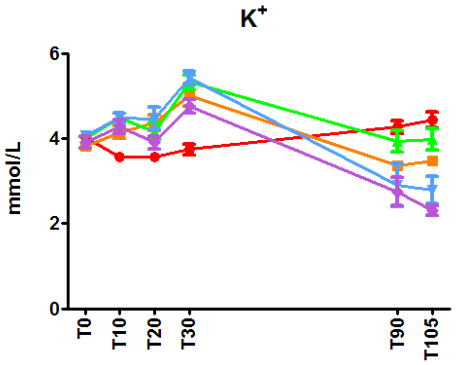
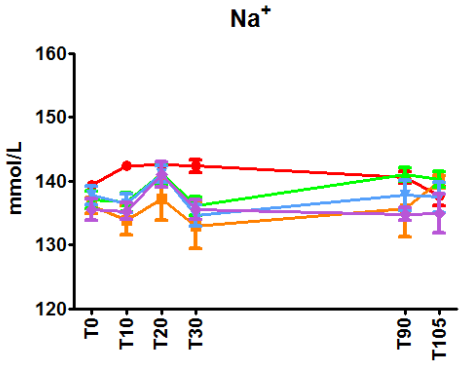
T105

D


K+
6
4
2
0
mmol/L
T0
T10
T20
T30
T90
T105

E


Na+
160
150
140
130
120
mmol/L
T0
T10
T20
T30
T90
T105

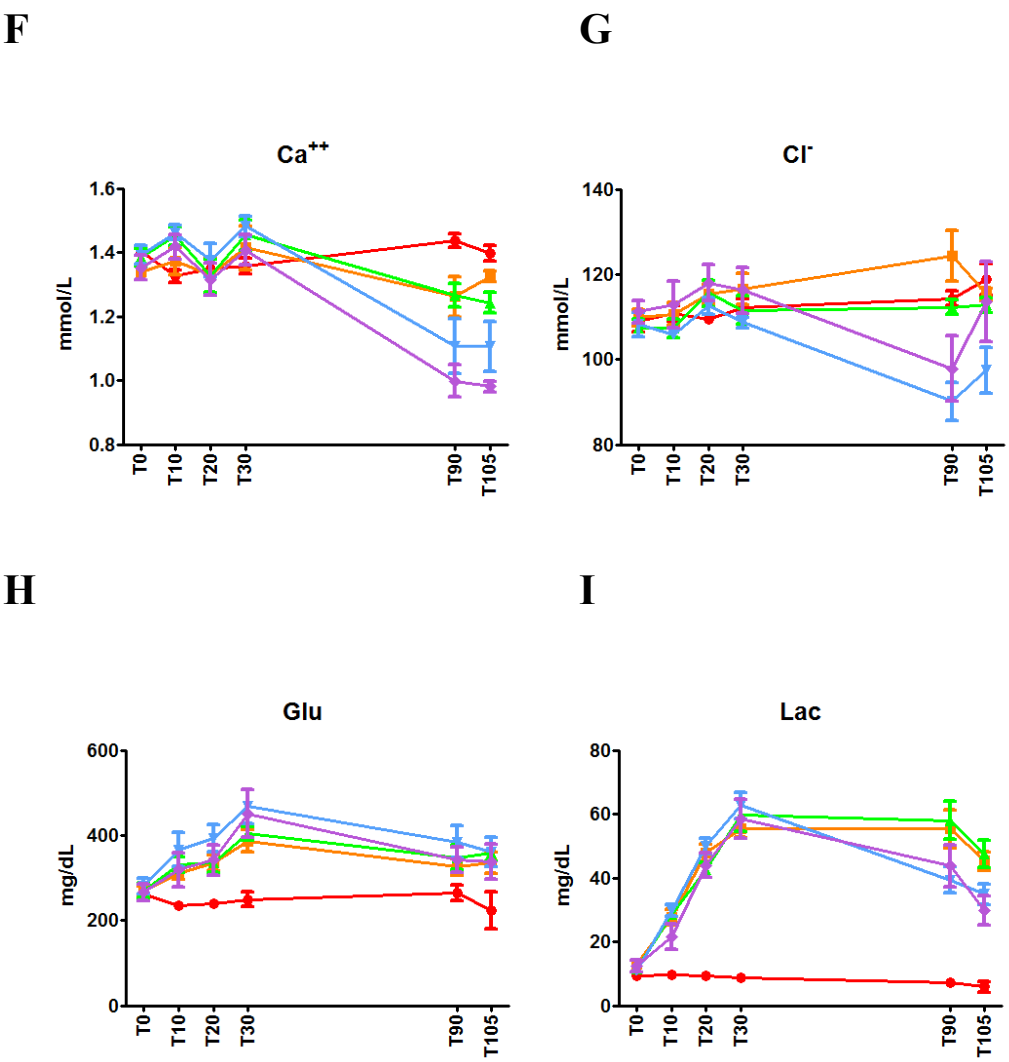


Figure 4.5. One-Hour Shock Study: Whole-blood parameter data.

Infusion of either LR (n=10), LR plus 4.3 mM Mel (n=10), 140 mM BHB with 1.5x10-6 mM Mel (n=10) or 140 mM BHB with 4.3 mM Mel (n=10) was achieved by administering three times the volume of blood lost per hour for one hour. Times on the x-axis reflect minutes. Data points are depicted as mean ± SEM.

Statistical differences in tHb were observed from T10 to T90 between sham-operated animals and all hemorrhaged groups (p<0.01). This is to be expected as the loss of blood is accompanied by a reduction in red cell mass(Carey, Lowery et

al. 1971). Furthermore, the infusion of large volumes of resuscitation fluids is accompanied by substantial hemodilution. A blood transfusion at T105 returned tHb to levels close to those of the starting point in hypovolemic animals.

Statistical differences in pH were only observed at T10 between shams and hemorrhaged animals ($p<0.05$). However, the mean values for all groups at all time points stayed relatively close to the physiologically neutral value of 7.4.

Table 4.4. One-Hour Shock Study: Whole-blood data.

					tHb (g/dL) Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	13.02 ± 0.78	A	12.17 ± 0.95	A	12.24 ± 0.77	A	11.83 ± 0.86	A	11.69 ± 1.54	A	8.35 ± 5.28	A
LR	12.06 ± 0.98	A	10.19 ± 0.52	B	8.63 ± 1.14	B	8.59 ± 0.44	B	5.81 ± 0.62	B	8.24 ± 0.60	A
LR/4.3 mM Mel	12.62 ± 0.45	A	10.37 ± 0.44	B	8.64 ± 0.80	B	8.80 ± 0.89	B	5.19 ± 0.49	B	7.79 ± 0.65	A
140 mM BHB/1.5x10-6 mM Mel	12.33 ± 0.78	A	10.42 ± 0.80	B	8.94 ± 0.68	B	8.89 ± 0.97	BC	6.68 ± 1.63	B	8.09 ± 1.42	A
140 mM BHB/4.3 mM Mel	12.79 ± 0.76	A	10.66 ± 0.80	B	7.94 ± 0.73	B	6.48 ± 3.32	C	5.64 ± 0.90	B	7.60 ± 1.12	A
					pH Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	7.40 ± 0.04	A	7.38 ± 0.08	B	7.38 ± 0.06	A	7.33 ± 0.16	A	7.27 ± 0.19	A	7.27 ± 0.14	A
LR	7.45 ± 0.04	A	7.53 ± 0.05	A	7.37 ± 0.15	A	7.38 ± 0.16	A	7.33 ± 0.08	A	7.36 ± 0.14	A
LR/4.3 mM Mel	7.43 ± 0.05	A	7.50 ± 0.06	A	7.42 ± 0.05	A	7.47 ± 0.12	A	7.33 ± 0.09	A	7.35 ± 0.11	A
140 mM BHB/1.5x10-6 mM Mel	7.41 ± 0.06	A	7.44 ± 1.11	AB	7.29 ± 0.20	A	7.34 ± 0.25	A	7.38 ± 0.16	A	7.38 ± 0.14	A
140 mM BHB/4.3 mM Mel	7.43 ± 0.06	A	7.50 ± 0.08	A	7.41 ± 0.08	A	7.46 ± 0.09	A	7.42 ± 0.05	A	7.44 ± 0.06	A
					sO_2 (%) Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	96.15 ± 2.80	A	95.99 ± 1.46	A	92.14 ± 4.21	B	94.90 ± 4.04	A	95.66 ± 2.59	A	96.51 ± 0.70	A
LR	89.24 ± 5.66	B	96.42 ± 0.99	A	96.11 ± 1.39	A	95.70 ± 1.21	A	93.43 ± 4.45	A	93.21 ± 4.46	A
LR/4.3 mM Mel	86.57 ± 4.23	B	95.31 ± 2.19	A	95.74 ± 0.82	A	95.68 ± 0.93	A	97.00 ± 2.28	A	94.34 ± 2.16	A
140 mM BHB/1.5x10-6 mM Mel	86.93 ± 2.61	B	95.86 ± 1.37	A	95.66 ± 0.71	A	94.91 ± 2.50	A	95.90 ± 1.00	A	96.01 ± 1.43	A
140 mM BHB/4.3 mM Mel	88.64 ± 3.45	B	96.00 ± 1.41	A	95.97 ± 1.32	A	96.03 ± 1.00	A	95.64 ± 0.77	A	95.45 ± 2.47	A
					K^+ (mmol/L) Mean ± SD							
	T0		T10		T20		T30		T90		T105	
Sham	4.02 ± 0.26	A	3.57 ± 0.33	B	3.56 ± 0.25	B	3.75 ± 0.42	B	4.26 ± 0.50	A	4.44 ± 0.52	A
LR	3.83 ± 0.29	A	4.14 ± 0.43	A	4.38 ± 0.53	A	5.01 ± 0.79	A	3.35 ± 0.32	AB	3.47 ± 0.26	BC
LR/4.3 mM Mel	4.00 ± 0.34	A	4.49 ± 0.28	A	4.17 ± 0.62	AB	5.32 ± 0.53	A	3.93 ± 0.78	AB	3.98 ± 0.73	AB
140 mM BHB/1.5x10-6 mM Mel	4.08 ± 0.33	A	4.49 ± 0.34	A	4.47 ± 0.78	A	5.41 ± 0.48	A	2.90 ± 1.41	B	2.79 ± 0.98	CD
140 mM BHB/4.3 mM Mel	3.90 ± 0.39	A	4.28 ± 0.44	A	3.90 ± 0.44	AB	4.75 ± 0.44	A	2.75 ± 0.80	B	2.30 ± 0.31	D

Na^+ (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	139.40 ± 1.65	A	142.36 ± 2.25	A	142.50 ± 1.96	A	142.36 ± 3.23	A	140.55 ± 2.95	A	137.63 ± 4.31	A
LR	136.13 ± 3.31	A	133.89 ± 6.86	B	137.13 ± 9.28	A	133.00 ± 10.96	B	135.70 ± 14.28	A	139.89 ± 3.14	A
LR/4.3 mM Mel	137.10 ± 4.28	A	136.70 ± 4.47	B	141.10 ± 5.55	A	136.10 ± 4.58	AB	141.00 ± 3.56	A	140.22 ± 3.70	A
140 mM BHB/1.5x10-6 mM Mel	137.78 ± 4.38	A	136.44 ± 4.33	B	141.11 ± 4.01	A	134.56 ± 5.10	AB	137.75 ± 6.52	A	137.44 ± 7.04	A
140 mM BHB/4.3 mM Mel	135.50 ± 4.84	A	135.25 ± 3.66	B	141.00 ± 5.45	A	135.50 ± 4.38	AB	134.83 ± 2.48	A	134.86 ± 8.09	A
Ca^{++} (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	1.40 ± 0.03	A	1.33 ± 0.06	B	1.36 ± 0.03	A	1.36 ± 0.07	A	1.43 ± 0.07	A	1.39 ± 0.06	A
LR	1.34 ± 0.07	A	1.37 ± 0.13	AB	1.33 ± 0.15	A	1.41 ± 0.21	A	1.26 ± 0.20	AB	1.33 ± 0.05	A
LR/4.3 mM Mel	1.39 ± 0.09	A	1.45 ± 0.09	A	1.33 ± 0.17	A	1.45 ± 0.15	A	1.27 ±0.12	AB	1.24 ± 0.10	AB
140 mM BHB/1.5x10-6 mM Mel	1.39 ± 0.09	A	1.46 ± 0.07	A	1.38 ± 0.15	A	1.48 ± 0.10	A	1.11 ± 0.24	BC	1.11 ± 0.23	BC
140 mM BHB/4.3 mM Mel	1.35 ± 0.11	A	1.42 ± 0.11	AB	1.32 ± 0.14	A	1.41 ± 0.14	A	1.00 ± 0.13	C	0.98 ± 0.04	C
Cl^- (mmol/L) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	109.00 ± 7.83	A	110.91 ± 6.04	A	109.50 ± 2.76	A	112.18 ± 7.29	A	114.36 ± 5.78	AB	118.89 ± 10.62	A
LR	109.88 ± 5.41	A	110.67 ± 7.89	A	115.50 ± 6.46	A	116.56 ± 10.90	A	124.40 ± 18.75	A	115.33 ± 4.56	AB
LR/4.3 mM Mel	107.50 ± 6.52	A	107.30 ± 7.06	A	115.60 ± 9.90	A	111.60 ± 10.63	A	112.30 ± 5.54	AB	112.89 ± 5.16	AB
140 mM BHB/1.5x10-6 mM Mel	108.22 ± 8.51	A	105.89 ± 2.42	A	112.67 ± 5.85	A	108.67 ± 3.84	A	90.13 ± 12.70	C	97.44 ± 16.33	B
140 mM BHB/4.3 mM Mel	111.25 ± 7.54	A	112.88 ± 15.55	A	118.00 ± 11.93	A	116.25 ± 15.06	A	97.83 ± 18.79	BC	113.57 ± 25.07	AB
Glu (mg/dL) Mean ± SD												
	T0		T10		T20		T30		T90		T105	
Sham	263.10 ± 23.85	A	234.36 ± 32.41	B	240.20 ± 35.12	B	249.18 ± 56.80	B	264.46 ± 59.72	B	224.00 ± 123.63	B
LR	268.74 ± 60.65	A	309.89 ± 41.62	AB	335.13 ± 52.27	AB	386.75 ± 75.19	A	325.75 ± 53.73	AB	336.25 ± 70.76	AB
LR/4.3 mM Mel	270.50 ± 45.73	A	331.63 ± 80.84	AB	336.88 ± 69.48	A	404.75 ± 42.71	A	348.00 ± 86.26	AB	359.71 ± 53.66	AB
140 mM BHB/1.5x10-6 mM Mel	280.88 ± 50.41	A	366.50 ± 11.34	A	393.63 ± 90.60	A	468.00 ± 114.73	A	384.75 ± 105.59	A	362.11 ± 102.88	A
140 mM BHB/4.3 mM Mel	266.25 ± 5.61	A	318.88 ± 114.38	AB	342.00 ± 94.68	A	450.67 ± 137.26	A	343.00 ± 67.48	AB	338.00 ± 103.27	AB

Lac (mg/dL) Mean ± SD	T0		T10		T20		T30		T90		T105	
Sham	9.50 ± 4.06	A	9.70 ± 3.92	B	9.40 ± 3.20	B	8.82 ± 2.52	B	7.30 ± 1.26	B	5.86 ± 4.38	C
LR	13.00 ± 3.07	A	27.67 ± 6.96	A	47.88 ± 7.53	A	55.56 ± 8.63	A	55.40 ± 18.70	A	45.33 ± 8.87	A
LR/4.3 mM Mel	11.50 ± 1.90	A	28.60 ± 3.13	A	42.60 ± 7.32	A	59.60 ± 15.72	A	58.00 ± 18.73	A	47.56 ± 13.17	A
140 mM BHB/1.5x10-6 mM Mel	10.78 ± 2.49	A	29.89 ± 5.40	A	50.00 ± 7.31	A	62.78 ± 12.41	A	39.43 ± 11.19	A	34.88 ± 9.25	AB
140 mM BHB/4.3 mM Mel	12.38 ± 5.04	A	21.63 ± 11.31	A	44.00 ± 11.16	A	58.57 ± 16.23	A	43.83 ± 16.02	A	29.86 ± 12.09	B

Statistical significance is denoted by the presence of different letters. Groups not sharing a letter are statistically different ($p<0.05$). Abbreviations: BHB-β-Hydroxybutyrate. LR-Lactated Ringer's. Mel-Melatonin.

At T0, sO_2 was somewhat decreased in all groups except shams. This could be a result of the anesthetic induction with isoflurane. The only other difference observed was at T20, when shams seemed to have slightly less oxygenated blood than shams. However, those differences were not of clinical concern.

Increased blood K^+ levels are common in hemorrhagic shock (Carey, Lowery et al. 1971). K^+ levels increased as blood loss occurred. Shams' circulating K^+ was lower from T0 to T30 but progressively increased. After the one-hour shock period, animals infused with either of the LR treatments maintained blood K^+ at levels close to baseline. From T90 to T105, both of the BHB/M groups had mean K^+ concentrations below the normal range of 3.9-9.2 mmol/L (Sharp and Villano 1998).

Hyponatremia occurs with hemorrhage (Carey, Lowery et al. 1971). Some differences were observed between shams and hemorrhaged animals at T10 and T30 ($p<0.05$). During the hemorrhagic phase, Na^+ levels in the hypovolemic groups seemed to parallel blood pressure. Through the one-hour shock period, circulating Na^+ returned to homeostatic levels. This is probably due to the Na^+ content in the solutions infused.

Though hemorrhage did affect circulating Ca^{++} levels, those changes were within the reference values (1.2-1.6 mmol/L) (Sharp and Villano 1998). However,

blood Ca^{++} fell below the normal range in animals administered either one of the isotonic BHB/M formulations used in this study. These differences were statistically significant at T90 ($p<0.05$) and T105 ($p<0.01$).

Cl^- levels fluctuated slightly during blood withdrawal, reaching levels above the normal range of 84-110 mmol/L (Sharp and Villano 1998) at T20. At T90, the administration of isotonic BHB/M formulations resulted in Cl^- concentrations on the low end of the reference values while animals infused with LR had circulating Cl^- levels above the normal range. A blood transfusion brought blood Cl^- to levels close to baseline in all hypovolemic groups.

Sham-operated animals had lower Glu and Lac levels than all other treatments ($p<0.05$) at all time points except T0, consistent with a normal response to shock (Carey, Lowery et al. 1971).

Blood Glu levels increased after T10 in hemorrhaged animals, as expected in a normal response to shock (Carey, Lowery et al. 1971).

Sham rats had lower blood Lac than all hemorrhaged groups from T10 to T105 ($p<0.01$). It should also be pointed out that the administration of LR, with or without melatonin, resulted in slightly higher circulating Lac than when the rats were infused with either one of the isotonic BHB/M solutions.

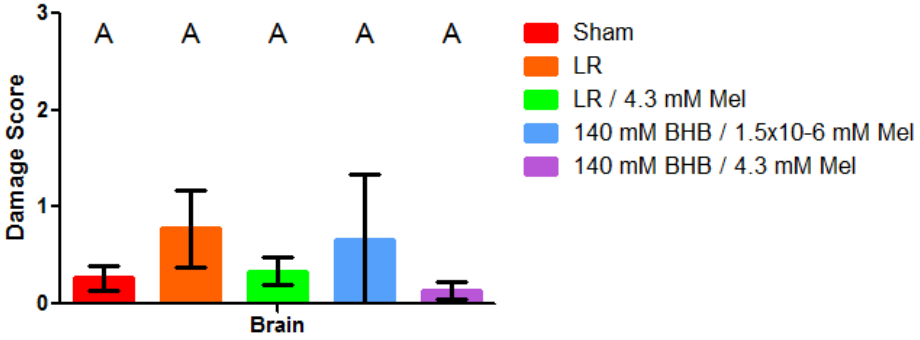
Sham rats were excluded from the comparisons between animals that lived to 10 days after 60% blood loss and those that died. All sham-operated animals survived to the 10 days endpoint. Hence their inclusion in these analyses could bias the data. tHb at T20 and Ca^{++} at T90 were lower in animals that died compared to those that lived to 10 days ($p<0.05$).

Sham-operated animals were also excluded from regression analyses. Low pH at T10 negatively influenced survival ($p<0.05$; HR=0); the opposite was observed at T30 ($p<0.05$; HR=0.03). Higher sO_2 at T90 resulted in lower survival ($p<0.05$; HR=1.78). Lower Glu at T0 ($p<0.05$; HR=0.96) and T105 ($p<0.05$; HR=0.98) ensued lower survival; a reversed trend was observed at T10 ($p<0.05$; HR=1.02) and T90 ($p<0.05$; HR=1.02).

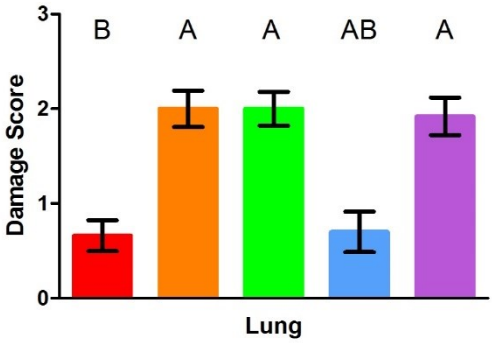
Histopathological Scoring

Histopathological damage scores can be graphically observed in Figure 4.6. Statistical differences where only observed in lung. Shams had less evidence of tissue damage than 10-day survivors administered LR, LR plus melatonin, or 140 mM BHB with 4.3 mM melatonin ($p<0.01$). It is possible that the lungs underwent some level of edema and congestion as a response to the fluid overload common to isotonic resuscitation. However, since the damage scores were only moderate, we can infer that the injury to the tissue is on its way to a successful resolution.

A


A
A
A
A
A
Damage Score
3
2
1
0
Brain
Sham
LR
LR / 4.3 mM Mel
140 mM BHB / 1.5x10-6 mM Mel
140 mM BHB / 4.3 mM Mel

B


B
A
A
AB
A
Damage Score
3
2
1
0
Lung

C

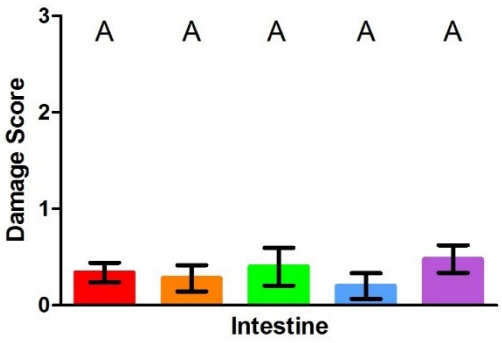


Figure 4.6. One-Hour Shock Study: Histopathological scores for (A) brain, (B) lung, and (C) small intestine.

Rats that survived to the experimental time point of 10 days were euthanized. Infusion of either LR (n=7), LR plus 4.3 mM Mel (n=6), 140 mM BHB with 1.5x10-6 mM Mel (n=1) or 140 mM BHB with 4.3 mM Mel (n=5) was achieved by administering three times the volume of blood lost per hour for one hour. All sham-operated animals (n=10) were included. Bars not present represent damage score values of 0. Data are depicted as mean ± SEM. Statistical significance is denoted by the presence of different letters.

CHAPTER V: DISCUSSION

In short, our experiments support an adjustment in the composition of the previously published hypertonic BHB/M which is intended to be infused as a low volume (1 ml/kg) resuscitation fluid. The ketone component of BHB/M, the D-stereoisomer of β-Hydroxybutyrate, should remain to be administered at a concentration that results in 4 mM circulating BHB. Melatonin can be lowered to a concentration a million-fold lower than the original concentration of 43 mM without affecting survival rate. Bringing melatonin to a lower level means the use of DMSO can be greatly reduced. This is advantageous since there is still much controversy over the use of DMSO (Davis 1984). We also demonstrated that a slow infusion after a bolus administration is not necessary. This is a highly desirable trait as it increases the feasibility for self-administration in warfare scenarios. Finally, administering BHB/M as an isotonic resuscitation fluid does not provide the same survival benefit as the standard of care.

In the first set of experiments, we observed that **a single 1 ml/kg bolus of BHB/M provides similar survival benefits as a bolus plus a slow infusion**. Previously, BHB/M had been administered as a 1 ml/kg bolus followed by a 100 µl/hr slow infusion (Klein, Wendroth et al. 2010). We decided to explore the feasibility of infusion BHB/M as a single bolus only. A single bolus is more practical in terms of self-administration. Our results show that administering

BHB/M as a single 1 ml/kg bolus had no statistically different survival compared to the administration of a 1 ml/kg bolus followed by a 100 µl/hr infusion for one hour. In fact, animals that were administered a single bolus lived longer than those in which a slow infusion was continued. The lack of statistical difference could be a result of a small sample size. However, there are a few mechanistic interpretations that could explain these results.

Osmolar overload and the potential of causing hyperosmolar comma is a concern when utilizing hypertonic fluids as a resuscitative strategy. Normal plasma osmolality ranges from 280 to 295 mOsm/l (Kreimeier and Messmer 2002). Plasma osmolalites higher than 320 mOsm/l can be tolerated if transient (Kramer 2003). However, if allowed to persist they can develop negative neurological consequences (Kreimeier and Messmer 2002; Kramer 2003). Other risks associated with the administration of hypertonic resustitation fluids include hypernatremia, hypokalemia, hemolysis, and increased clotting times, among others (Kreimeier and Messmer 2002).

The organism has an innate capacity to compensate for acute changes in plasma osmolarity and electrolyte imbalance. Per contra, chronic alterations may overwhelm the body's capacity to countervail physiological alterations. A single bolus potentially allows the organism to balance the osmolarity and electrolyte

content of the blood. Continuing to infuse a hypertonic solution may exceed the individual's ability to compensate for the osmolar load and electrolyte imbalance.

We also demonstrated that **it is important to maintain circulating BHB at a 4 mM concentration in order to obtain maximum survival**. Our data supports maintaining BHB at a 4 M concentration when administered at a volume of 1 ml/kg. Our collaborators conducting the pig model experiments have observed similar survival benefits when administering 2 M BHB at 2 ml/kg (data not shown). Empirically, it can be inferred that both modes of administration would result in an overall bodily concentration of 4 mM BHB. This assumption is supported by our data on plasma BHB (Figure 3.13).

The BHB concentration is responsible a fluid shift from the intracellular space into the intravascular space. A 4 M BHB solution contains 8,000 mOsm/l; 4,000 mOsm/l from the BHB itself and 4,000 mOsm/l from the sodium content as we use the sodium salt form of BHB. The administration of a hypertonic solution results in an osmotic gradient that rapidly mobilizes intracellular water into the intravascular space (Guyton and Hall 2001; Kreimeier and Messmer 2002), restoring MAP and hence CO and perfusion (Kreimeier and Messmer 2002; Kramer 2003). Reducing the concentration of circulating BHB would result in a decrease of the plasma expansion attributed to the hypertonic effect. However, volume

kinetics experiments would be required to confirm this theory by quantifying the changes in the distribution of bodily fluids.

BHB is also important in the maintenance of ATP production. BHB can be enzymatically converted into two molecules of acetyl-CoA which can directly enter the Krebs cycle and maintain energy production (Kashiwaya, Takeshima et al. 2000). Administering BHB at lower concentrations would issue a direct decline in ATP availability; reducing the concentration by 10-fold would be insufficient to increase plasma BHB above the baseline circulating levels (~0.4 mM) observed in our study (Figure 3.13) and by Klein et al (2010).

A 4 M NaCl solution also contains 8,000 mOsm/l; 4,000 from the sodium and 4,000 from the chloride. However, **a 1ml/kg bolus of 4 M NaCl could not support survival as effectively as a 1ml/kg bolus of 4 M BHB**. Since NaCl does not provide any nutritional support, it only has the circulatory benefits common to hypertonic resuscitation fluids. It does not have an effect on ATP production, like BHB does, which is essential for the maintenance of normal or near normal cellular function.

It would be interesting to investigate concentrations higher than 4 M BHB in the composition of BHB/M, however, 4 M is already in the upper limits of the

water solubility of the compound and in order to conduct those experiments a new approach for diluting the components would be required.

In our rat model of hemorrhagic shock, **melatonin provides therapeutic effects at very low concentrations**. The infusion of a 1 ml/kg bolus of 4 M BHB with melatonin concentrations a million-fold lower than the previously published formulation (Klein, Wendroth et al. 2010) still supported survival (Figure 3.5). Melatonin peaks in rats are ~8.61×10^{-7} mM (Benot, Molinero et al. 1998). Hence, administering a 1 ml/kg bolus of 43 mM melatonin, the way it occurred in the experiments by Klein et al (2010), would result in plasma levels almost fifty thousand times higher than peak, oversaturating the system.

Though the concentration of melatonin can be greatly reduced, it cannot be eliminated for we have evidence that **melatonin is important for survival**. 4 M BHB without the addition of melatonin resulted in the same survival 10-day survival (30%) as 4 M NaCl with melatonin (Figure 3.5). As stated above, 4 M NaCl does not provide nutritional support. However, our NaCl control group had 0.000043 mM melatonin included in its composition. There is evidence that supports the assumption that having melatonin in its formulation would provide ROS scavenging and anti-inflammatory effects (Cuzzocrea and Reiter 2001; Mayo, Sainz et al. 2005) and hence a survival benefit. When BHB was administered

without melatonin, initial survival was observed, but it could not be supported long term. This provides evidence that melatonin may be responsible for the long term survival effects of BHB/M.

A minor aspect of our experiments aimed at determining if the active pharmacological induction of hypothermia enhanced survival. Passive cooling was an important component of the studies by Klein et al (2010). It is possible that the mechanism by which hypothermia provides a therapeutic effect relies on the fact that a single °C reduction in body temperature can result in a 6 to 10% drop in metabolic rate (Polderman 2009). Decreasing metabolic rate lowers the individual's energy and oxygen requirements.

In hibernation, a reduction in body temperature occurs as the result of decreased metabolic rate (Heldmaier, Klingenspor et al. 1999; Ortmann and Heldmaier 2000), not the other way around. It has been suggested that the decline in metabolic rate in hibernators may be related to changes in circulating thyroid hormone since thyroid hormone is a major determinant of an organism's overall metabolic rate. 3-iodothyronamine (T1AM) is a recently discovered derivative of thyroxine (T4), the predominant form of thyroid hormone, is found in the brain and blood of Siberian hamsters, a hibernating species (Piehl, Heberer et al. 2008).

Scanlan et al (2004) showed that intraperitoneal injections of T1AM rapidly lower body temperature and slow heart rate in mice. T1AM at 50 mg/kg lowered body temperature from 37° to 31°C within 30 minutes. These mice's phenotype mimicked natural hibernation for they were inactive, in a curled position, and cool to the touch; this phenotype remained for 6 to 8 hours after injection.

In our rat experiments, a slight reduction in temperature was observed in normotensive animals administered T1AM intravenously at either 50 mg/kg, 25 mg/kg, 10 mg/kg, 2 mg/kg, or 0 mg/kg. However, **administration of T1AM in normotensive rats did not influence temperature more than anesthesia** (Figure 3.9). We believe that the use of an anesthetic could be concealing the hypothermic effects of T1AM, if any. In order to confirm these suspicions, consideration was given to conducting these experiments without anesthesia. However, despite T1AM been known to induce a torpor-like state which is also consistent with a response to anesthesia, Scanlan et al (2004) do not report data on analgesia. Hence, though an animal may seem unconscious, they may still be able to experience pain. Considering the level of agony and distress the surgical procedure causes to the animals, we do not find it reasonable to execute the surgeries without an anesthetic agent.

We decided not to explore the possibility of adding T_1AM to BHB/M because of the results described above and due to statistical concerns. In our previous experiments we have found no statistical differences in survival (p>0.05) between sham-operated animals and hypovolemic rats administered both 4 M BHB and melatonin at any concentration (Figure 3.5). Animals that were administered BHB without melatonin or melatonin with NaCl observed survival statistically lower (p<0.05) than shams. Not observing statistically significant variation in survival between our different treatment groups for the melatonin dose-ranging study and the shams complicates determining if the adjunction of T1AM to BHB/M could improve outcomes of hemorrhagic shock since, with the current statistical tools and tests available, establishing differences between the effects of BHB/M with and without T1AM is not likely considering there are already no differences between shams and the different formulations of BHB/M tested.

We decided to evaluate the efficacy of BHB/M as an isotonic formulation. We found that **the isotonic form of BHB/M is not efficient for sustaining survival when compared to the standard of car (LR)**. It was necessary for us to conduct isotonic experiments because the standard of care for hemorrhagic shock is the administration of isotonic non-sanguineous resuscitation fluids (Falk, O'Brien et al. 1992); the use of hypertonic solutions is still contentious and not widespread

(Kreimeier and Messmer 2002). Isotonic solutions need to be administered in large volumes because their electrolyte content allows for water to move freely between the intravascular compartments (Moon, Hollyfield-Gilbert et al. 1994). Furthermore, infusing abundant quantities of fluid may result in fluid accumulation and edema, particularly in the lung (Imm and Carlson 1993). There is no "ideal" resuscitation fluid currently available, and though LR is very effective, many researchers are still focusing on the development of the resuscitative strategy that is effective at small infusion volume, can be administered rapidly, has sustained effects, and is safe (Kyes and Johnson 2011).

In our rat model of hemorrhagic shock, LR administration was more efficient at sustaining survival after 60% blood loss both at 24 hours and 10 days after surgery than any other isotonic treatment used in these experiments (Figure 4.2). This may be because parenteral fluids should have a similar composition to normal plasma (Hartmann 1934). LR provides essential mineral constituents of blood which isotonic BHB/M does not.

Although we did not find any electrolyte effects on survival, LR, with or without melatonin, provided better electrolyte support than 140 mM BHB with $1.5x10^{-6}$ mM Mel or 140 mM BHB with 4.3 mM Mel as evident by the fact that the treatments containing LR maintained higher values for K^+, Na^+, Ca^{++}, and Cl^-,

regardless of statistical significance. (Figure 4.5) We speculate that the administration of a mixture of electrolytes that is similar to that of the plasma is important for sustained survival when the resuscitation strategies involve isotonic fluids.

In an effort to identify whether physiological constants (MAP, HR, and rectal temperature) whole-blood (tHb, pH, sO_2, pO_2, pCO_2, K^+, Na^+, Ca^{++}, Cl^-, Glu, and Lac), and plasma (BHB and TNF-α) parameters measured during the hemorrhagic shock protocol that could serve as a predictor of survival we conducted analyses comparing 10-day survivors to non-survivors. We also ran Cox Proportional Hazards regression models expecting to identify relationships between survival and all other data. We found that **physiological constant, whole-blood, and plasma parameter data do not provide information that can consistently help predict survival**. Though some statistically significant observations were made, they were not consistent through the shock procedure. Furthermore, they were often contradictory from one time point to the next. It is possible that none of the parameters measured particularly affect survival individually.

Melatonin has been suggested to have anti-inflammatory effects (Cuzzocrea and Reiter 2001; El-Shenawy, Abdel-Salam et al. 2002; Mathes, Kubulus et al. 2008; Wu, Tsou et al. 2008). It has been shown to reduce tissue damage (Reiter,

Calvo et al. 2000; Cuzzocrea and Reiter 2001; Yang, Subeq et al. 2011) and prevent multiple organ failure (Crespo, Macías et al. 1999; Wu, Tsou et al. 2008). One of the proposed mechanisms for this protection is based on evidence suggesting melatonin can prevent the expression of adhesion molecules (Cuzzocrea and Reiter 2001; Kang, Ahn et al. 2001).

In the hypertonic resuscitation experiments, our histological results showed that the lowest dose of melatonin administered, 0.000043 mM, had statistically higher injury scores in small intestine than the one administered the highest dose of 4.3 mM in our second melatonin dose-ranging study. Furthermore, the melatonin response observed at T30 in TNF-α levels also support the idea of a dose-dependent anti-inflammatory action of melatonin. Hence **melatonin may have dose-dependent anti-inflammatory properties**. It is possible that other dose-dependent differences were masked by statistical variability, unequal variances, and low sample size. However, these differences do not seem to affect survival, supporting a reduction in the concentration of melatonin in the composition of BHB/M in a rat model.

Surviving to 10 days following 60% blood loss is no guarantee of indefinite survival. Many successfully resuscitated patients may have delayed deaths (Deitch 1992; Fink, Hayes et al. 2008) as the inflammatory and immunologic alterations

triggered by trauma and hemorrhage may lead lead to a series of complications known as multiple organ failure (MOF) (Yao, Redl et al. 1998). In MOF, organ injury is not directly caused by exogenous factors (Deitch 1992), instead, it is the result of disproportionate self-destructive inflammation (Roumen, Hendriks et al. 1993; Yao, Redl et al. 1998; Rotstein 2000). In this condition, the host's own immune system engages in an actively destructive process. The immunological conditions leading to tissue damage include, amongst others, release of pro-inflammatory cytokines and changes in endothelial permeability (Ayala, Wang et al. 1991; Deitch 1992; Deitch, Xu et al. 1994; Martin, Boisson et al. 1997; Yao, Redl et al. 1998; Meng, Dyer et al. 2000; Keel and Trentz 2005; Yang, Subeq et al. 2011).

MOF is unique in the sense that it can lead to the malfunction of organs not involved in the original traumatic insult and that days to weeks can pass before the compromise of organs not involved in the original insult (Deitch 1992; Roumen, Hendriks et al. 1993). It is the leading cause of death in surgical intensive care units, accounting for 50-80% of the unit's defunctions (Deitch 1992; Roumen, Hendriks et al. 1993; Rotstein 2000).

We conducted histopathological analyses to estimate whether our animals were likely to exhibit deaths due to MOF past the 10 days they were monitored.

Since our post-operatory monitoring is not comprised of weeks, observing micro anatomical changes in different tissues provides information regarding the health status of the experimental subjects that survived the entire 10 days and helps us identify whether those animals would have kept living indefinitely or were likely to suffer from MOF and die in the near future. The central nervous system remains the major single limiting organ after injury. Furthermore, the "gut hypothesis" states that ischemia causes changes in the permeability of the intestine that alters the immunological function of the gut and increases systemic translocation of bacteria, leading to the development of a systemic inflammatory response syndrome (SIRS), followed by an acute respiratory distress syndrome (ARDS), and finally MOF (Baue, Faist et al. 2000). For these reasons, we decided to examine the brain, intestine, and lungs.

In both the hypertonic and the isotonic experiments, the highest average damage scores were only moderate, suggesting that the injury to the tissues was on its way to a successful resolution. Based on these results it appears that **BHB/M reduces the risk of Multiple Organ Failure.**

To recapitulate, BHB/M is successful at achieving 10-day survival in rats hemorrhaged to 60% of their calculated blood volume through immediate, short-term, and long term mechanisms. The immediate effects can be attributed to its

hyperosmolarity; increases in the solute concentration of the blood shifts intracellular water intro the intravascular space, increasing MAP, CO, and systemic perfusion. In the short term, BHB provides nutritional support allowing for the maintenance of energy production and cellular function; melatonin scavenges for potentially damaging ROS. It can be inferred that the long-term effects of BHB/M are associated with the immunomodulatory effects of melatonin.

Future Directions

Subsequent experiments should be aimed at identifying the mechanisms, beyond speculation, by which rats hemorrhaged to 60% of their calculated blood volume achieve 10 day survival after a 1 mg/kg infusion of BHB/M. Thus far, we do not have any evidence to substantiate our proposed mechanisms of action of BHB/M.

Volume kinetics experiments would provide information regarding changes in the distribution of bodily fluids after infusion. This would either confirm or refute the idea that BHB/M mobilizes intracellular water into the intravascular space. Additionally, they would offer a quantitative analysis of volume and rate of plasma expansion plus data on distribution and elimination of fluids.

Special attention should be paid to the protective mechanisms of melatonin, particularly to those involving the immune function. It is well accepted that melatonin has immunomodulatory roles (Mayo, Sainz et al. 2005). However, we do not know where in the inflammatory cascade melatonin exerts its effects in the hemorrhagic shock scenario. Does it reduce pro-inflammatory cytokine production and/or increase anti-inflammatory cytokine production? Does it affect the expression of adhesion molecules? Does it interfere with leukocyte-endothelium interactions?

Our assessment of plasma TNF-α levels could be expanded to include other pro-inflammatory cytokines, such as IL-1 and IL-6, as well as anti-inflammatory cytokines, like IL-4 and IL-10. Furthermore, their values should not be assessed individually as a strong pro-inflammatory response may be counteracted by a strong anti-inflammatory response.

Increases in pro-inflammatory cytokines triggers the expression of endothelial adhesion molecules. The adhesion of neutrophils to the endothelium plays an important role in the inflammatory response. During inflammation, endothelial cell-leukocyte interactions are involved in tissue injury. Adhesion molecules are responsible for the interplay between cells of the endothelium and neutrophils resulting in their migration from the intravascular space to tissues (Yao,

Redl et al. 1998; Dayal, Hasko et al. 2002). These adhesion molecules include selectins and integrins (Yao, Redl et al. 1998; Dayal, Hasko et al. 2002; Tizard 2004). Selectin-mediated binding is weak and transient (Tizard 2004); it initiates the interaction between leukocytes and the endothelium (Angle, Hoyt et al. 1997). Integrin-mediated binding is strong (Tizard 2004) and coordinates extravasation (Angle, Hoyt et al. 1997). The primary adhesion molecules on polymorphonuclear (PMN) cells are L-selectin and integrin subunits CD11a, CD11b, and CD18; on the endothelium, P-selectin and intercellular adhesion molecule-1 (ICAM-1) are the main adhesion molecules expressed (Yao, Redl et al. 1998; Dayal, Hasko et al. 2002; Tizard 2004). Integrin expression on hemorrhaged-rat neutrophils from peripheral blood can be quantified using flow cytometry. Additionally, adhesion can be assessed *in vitro* in a quantitative endothelial cell-neutrophil adhesion assay (Barouch, Miyamoto et al. 2000).

The effects of melatonin in the regulation of the solute content of the plasma should be investigated. Recently, melatonin has been suggested to have osmoregulatory effects in both terrestrial vertebrates and fish (Kulczykowska 2001). It has been suggested that melatonin can reduce glomerular filtration rate (GFR) (Tsuda, Ide et al. 1995) and urinary K^+, Na^+, and Cl^- excretion (Koopman, Minors et al. 1989), increasing plasma osmolarity. It is possible that the

administration of BHB/M regulates plasma expansion not only through a physicochemical mechanism involving an osmolar gradients but also through physiological mechanisms involving renal function.

Melatonin has also been implicated in circadian changes in blood pressure. In normal physiological function, blood pressure dips during sleep (Kryger, Roth et al. 2010). Melatonin production has been reported to be impaired in human patients with nocturnal hypertension. Non-dipping hypertensive individuals showed a reduction in nocturnal blood pressure after melatonin supplementation (Grossman, Laudon et al. 2006; Paulis and Simko 2007; Simko and Paulis 2007). Considering blood pressure is greatly reduced in hemorrhagic shock, it may seem paradoxical that the administration of melatonin would be beneficial to the bleeding individual. However, melatonin peaks during the dark phase of the day regardless of whether an animal is nocturnal or diurnal (Arendt 1995). It is possible that the nighttime effects of melatonin in diurnal mammals (e.g. humans) is opposite to those in nocturnal animals (e.g. rats). Perhaps a different rodent model of hemorrhagic shock is warranted in order to observe the effects of BHB/M in a diurnal mammal. The Sudanian grass rat (*Arvicanthis ansorgei*) has been used in numerous experiments comparing responses of nocturnal and diurnal rats (Dardente, Klosen et al. 2002; Dardente, Menet et al. 2004; Tournier, Dardente et

al. 2007) and could potentially offer a better rodent model of hemorrhagic shock, particularly when the objective is the development of therapies for human use.

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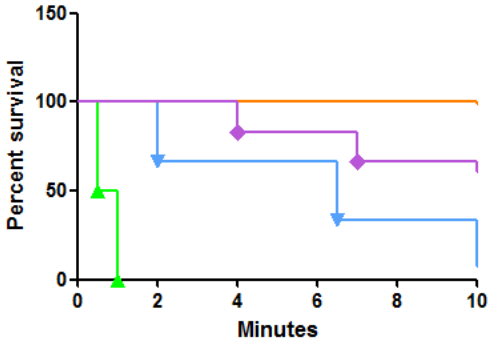
APPENDICES

Appendix I: Blood Volume Experiments

Four different formulas were compared before deciding which one to use to calculate blood volume (ml): 1) 6% of total body weight (g), 2) 6.5 % of total body weight, 3) 7% of total body weight, and 4) 6% of total body weight plus 0.77 ml. Acute (no blood return) surgeries were conducted in which animals were bled to 60% of their calculated blood volume based on the formulas above. Mean survival times in minutes were 740.50 ± 225.75, 6.50 ± 2.60, 0.75 ± 0.25, and 74.17 ± 49.30, respectively (Figure I.1).

A



B

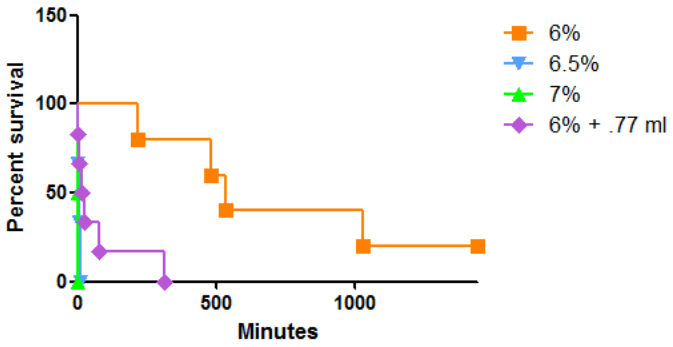


Figure I.1. Blood Volume Calculation: Kaplan-Meier plot of animals subjected to 60% blood loss at (A) 10 minutes and (B) 1440 minutes (24 hours).

Rats were hemorrhaged to 60% of their calculated blood volume in ml based on 6% of their total body weight (n=5), 6.5% of their total body weight (n=3), 7%of their total body weight (n=3), or 6% of their total body weight plus 0.77 ml (n=6). . Times on the x-axis reflect minutes after achieving 60% blood loss. Some lines may be indistinguishable due to overlap.

For our experiments, we decided to use the fourth formula, 6% of total body weight plus 0.77 ml, for numerous reasons. Firstly, when hemorrhaging animals to 60% of their total blood volume calculated as 6% of the total body weight, the rats' survival surpassed expectations. In fact, some individuals had to be censored after 24 hours of monitoring. Considering these individuals did not have any resuscitative strategies applied to them, it would be difficult to assess the benefit of a resuscitation fluid and/or a blood transfusion since they seemed to be able to compensate for the hemorrhagic insult using only innate responses. However, when using 6.5% or 7% of the total body weight to calculate blood volume, the insult was

so great that the animals were dying too fast. Secondly, larger individuals have smaller blood volumes relative to their size than smaller animals. The 6% of total body weight plus 0.77 ml formula takes this into account. Finally, we found that when hemorrhaging rats to 40% of their calculated blood volume using this formula we consistently achieved a MAP of 25 mmHg (Figure I.2).

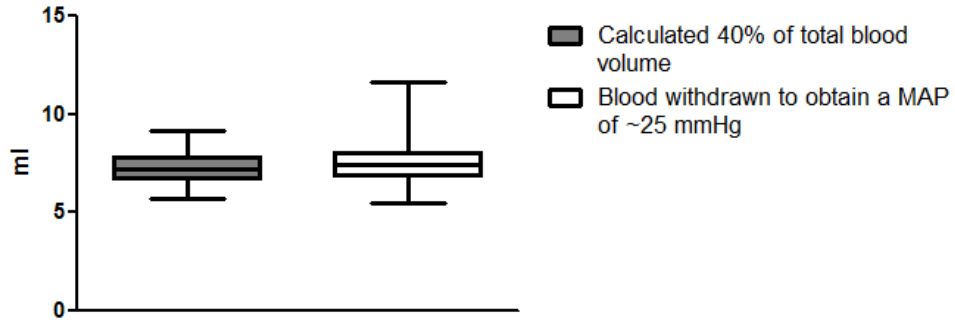


Figure I.2. Blood Volume Calculation: Volume (ml) comparisons between the calculated blood volume using the 6% of total body weight (g) plus 0.77 ml formula and the blood withdrawn in order to obtain a MAP of 25 mmHg.

The left column represents 40% of the calculated blood volume. The right column represents the volume recorded when animals reached a MAP of 25 mmHg (n=310). The line in the middle of the columns represents the mean. Error bars represent ranges.

Appendix II: Tissue Processing Protocol

This protocol was ran in a Tissue-Tek VIP 1000 tissue processor (Model 4617. Miles Laboratories Inc. Naperville, IL).

1. Bathe in 10% formalin for 30 minutes.
2. Bathe in 50% ethanol for 45 minutes.
3. Bathe in 70% ethanol for 45 minutes.
4. Bathe in 95% ethanol for 45 minutes.
5. Bathe in clean 95% ethanol for 45 minutes.
6. Bathe in 100% ethanol for 45 minutes.
7. Bathe in clean 100% ethanol for 45 minutes.
8. Bathe in xylene for 45 minutes.
9. Bathe in clean xylene for 60 minutes.
10. Bathe in paraffin for 30 minutes.
11. Bathe in clean paraffin for 30 minutes.
12. Bathe in clean paraffin for 30 minutes.
13. Bathe in clean paraffin for 30 minutes.

Appendix III: Hematoxylin and Eosin Staining Protocol

This protocol was ran in an Autostainer XL (Australian Biomedical Corporation Ltd. Melbourne, Australia).

1. Bake in the oven for 30 minutes.
2. Dip in xylene for 5 minutes.
3. Dip in xylene for 5 minutes.
4. Dip in 100% ethanol for 2 minutes.
5. Dip in 100% ethanol for 2 minutes.
6. Dip in 70% ethanol for 2 minutes.
7. Wash in running deionized water for 2 minutes.
8. Dip in hematoxylin for 5 minutes.
9. Wash in running deionized water for 2 minutes.
10. Dip in 2% glacial acetic acid for 4 seconds.
11. Wash in running deionized water for 2 minutes.
12. Dip in 2% ammonia water for 10 seconds.
13. Wash in running deionized water for 2 minutes.
14. Dip in 95% ethanol for 1 minute.
15. Dip in eosin for 1 minute.
16. Dip in 95% ethanol for 1 minute.

17. Dip in 100% ethanol for 2 minutes.

18. Dip in 100% ethanol for 2 minutes.

19. Dip in xylene for 2 minutes.

20. Dip in xylene for 2 minutes.

Appendix IV: D-BHB Assay

Absorbance values were read in a SpectraMax Plus384 plate reader (Molecular Devices, LLC. Sunnyvale, CA).

Software Setup

Wavelength	505 nm
Temperature	37°C
Mode	End Point

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Stnd01 10.417 mg/dL	Stnd02 5.209 mg/dL	Stnd03 2.604 mg/dL	Stnd04 0 mg/dL	Ctrl							
B	Unknown 1 (in triplicate)											
C	Unknown 2 (in triplicate)											
D	Unknown 3 (in triplicate)											
E	Etc…											
F												
G												
H												

Procedure

1. Place 107.5 µl of Enzyme R1 on all wells to be used
2. Incubate for 3 minutes at 37°C
3. Add standard, control, and sample volumes to respective wells. Follow volume guidelines:

Standard 01	3 µL	BHB Standard
Standard 02	1.5 µL	BHB Standard
Standard 03	0.75 µL	BHB Standard
Standard 04		
Control	3 µL	Distilled water
Unknowns	3 µL	Sample

4. Immediately measure at 505nm (Read 1)
5. Add 18 µL of Catalyst R2
6. Wait 5 minutes
7. Measure at 505 nm (Read 2)

Calculation

$$\text{BHB (mM)} = \frac{\text{Read 2}_{\text{Unknown}} - \text{Read 1}_{\text{Unknown}}}{\text{Read 2}_{\text{Standard}} - \text{Read 1}_{\text{Standard}}}$$

Note: Large concentrations might need to be diluted with distilled water. If diluted, calculation should be adjusted by multiplying the results by the dilution factor used.

Appendix V: TNF-α Assay

Absorbance values were read in a SpectraMax Plus384 plate reader (Molecular Devices, LLC. Sunnyvale, CA).

Software Setup

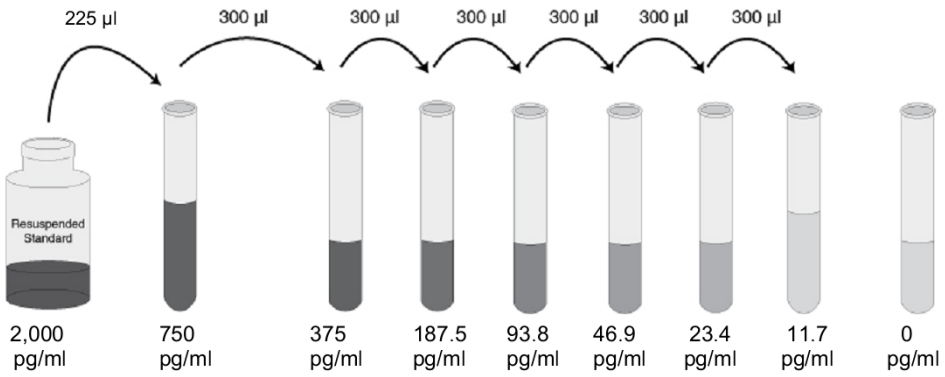
Wavelength	450 nm
Temperature	Room
Mode	End Point

Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Stnd 001 750 pg/ml		Unknown 001									
B	Stnd 002 375 pg/ml		Unknown 002									
C	Stnd 003 187.5 pg/ml		Unknown 003									
D	Stnd 004 93.8 pg/ml		Etc.									
E	Stnd 005 46.9 pg/ml											
F	Stnd 006 23.4 pg/ml											
G	Stnd 007 11.7 pg/ml											
H	Stnd 008 0 pg/ml											

Procedure

1. Bring all solutions to room temperature.
2. Create Standard Solutions.
    a. Fill one tube with 375 µL of Standard Diluent Buffer.
    b. Fill seven tubes with 300 µL of Standard Diluent Buffer.
    c. Create standard dilutions as explained in the diagram below.



3. Prepare samples.
    a. Dilute 20 µL of sample in 40 µL of Standard Diluent Buffer
    b. Dilute 60 µL of the sample/standard diluent buffer solution in 180 µL of Incubation Buffer
4. Add 100 µL of standard dilutions (From Step 2) to appropriate wells in duplicates.
5. Add 100 µL of diluted samples (From Step 3) to appropriate wells in duplicates.
6. Cover plate and incubate in the dark for *2 hours* at room temperature.

7. While waiting, dilute Wash Buffer 1:24. Final volume depends on amount to be used.
8. After the 2 hour wait, decant wells.
9. Wash wells *4 times*.
    a. Add 400 µL of Wash Buffer to all wells.
    b. Allow to soak for 15 to 30 seconds.
    c. Decant.
    d. Repeat
10. Add 100 µL of Rt TNF-α Biotin Conjugate to all wells being used.
11. Cover plate and incubate in the dark for *1 hour* at room temperature.
12. While waiting, make Streptavidin-HRP Working Solution.
    a. Dilute 120 µL of Streptavidin-HRP Concentrate in 12 mL of Streptavidin-HRP Diluent.
13. After the 2 hour wait, decant wells.
14. Wash wells *4 times* (see Step 9).
15. Add 100 µL of Streptavidin-HRP Working Solution to all wells being used.
16. Cover plate and incubate in the dark for *30 min* at room temperature.
17. After the 30 min wait, decant wells.
18. Wash wells *4 times* (see Step 9).
19. Add 100 µL of Stabilized Chromogen to all wells being used.
20. Cover plate and incubate in the dark for *30 min* at room temperature.

21. Add 100 µL of Stop Solution to all wells being used.
22. Mix plate.
23. Read absorbance at 450 nm.
24. Generate standard curve
25. Read concentrations from the standard curve.
26. Multiply values obtained by 6 to correct for dilutions in Step 3.