Characterization and Optimization of D-β-Hydroxybutyrate and Melatonin, a Treatment for Hemorrhagic Shock

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Dedication

To my parents.

Abstract

Hemorrhagic Shock is the leading cause of preventable death after trauma. A combination of 4 M D-β-hydroxybutyrate and melatonin (BHB/M) improves survival in preclinical hemorrhagic shock models. This dissertation is concerned with the characterization and optimization of this promising treatment. We conducted multiple experiments to evaluate the safety, optimize the dosing, administration and formulation, and to unravel the mechanism of action behind BHB/M. Our results indicate that intravenous infusion is the preferred route of administration, while intraosseous infusion was associated with decreased drug serum concentrations and increased mortality in a porcine hemorrhagic shock and trauma model. We also showed that lowering the melatonin concentration in combination with 4 M BHB decreased the efficacy of the treatment. Another objective was to define the maximum tolerated dose of 4 M BHB/43 mM M in porcine hemorrhagic shock with polytrauma. Our experiments indicate that administering increased volumes of 4 M BHB/43 mM M increased mortality in pigs exposed to hemorrhagic shock and injury. Death was likely a result of the interplay of hemorrhagic shock, injury, and BHB/M-induced hypernatremia and fluid translocation. Adverse effects were reversible in the surviving 2x 4 M BHB/43 mM M-treated pigs, and we concluded that this is the maximum tolerated dose of the treatment. Dimethyl sulfoxide (DMSO), used to solubilize melatonin in the original treatment solution, may exert adverse effects. We therefore developed and evaluated two novel BHB/M formulations void of DMSO. These formulations were lyophilized to increase drug stability and simplify drug preparation in the field. Treatment efficacy was retained in a rat hemorrhagic shock model, and novel formulations induced significantly lower hemolysis than the original formulation. Lastly, we conducted experiments to unravel the mechanism of action behind BHB/M in an *in vitro* ischemia/reperfusion model. We hypothesized that BHB/M increases survival of hemorrhagic shock and trauma by improving mitochondrial function during blood loss and resuscitation. However, treatment with BHB/M did not induce significant differences in ATP levels, mitochondrial ROS production or respiration in H9c2 cells exposed to oxygen glucose deprivation and reoxygenation, suggesting that treatment effects may not be mediated through changes in mitochondrial function or oxidative stress.

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List of Abbreviations

4-HNE	4-hydroxynenal
AA	antimycin A
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
AFMK	N1-acetyl-N2-formyl-5-methoxykynuramine
Alk Phos	alkaline phosphatase
AL	adenosine lidocaine
ALM	adenosine lidocaine magnesium
ALT	alanine aminotransferase
AMK	N1-acetyl-5-methoxykynuramine
ANT	adenine nucleotide translocase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
B	baseline
BDH1	D-β-hydroxybutyrate-dehydrogenase
BHB	D-β-hydroxybutyrate
BUN, SUN	blood urea nitrogen
C3HOM	cyclic 3-hydroxymelatonin
CARS	compensatory anti-inflammatory response
CCCP	carbonyl cyanide m-chlorophenyl hydrazine
CI	confidence interval
CK	total creatinine kinase
CLIA	Clinical Laboratory Improvement Amendments
C_{Max}	peak serum concentration

CO	cardiac output
CYP1A2	cytochrome P450 1A2
DADLE	D-ala^2-leu^5-enkephalin
DMSO	dimethyl sulfoxide
DO_2	oxygen delivery
DSC	differential scanning calorimetry
ECAR	extracellular acidification rate
EPR	emergency preservation and resuscitation
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FDA	food and drug administration
FDP	fructose 1,6-diphosphate
FiO2	inspired fraction of oxygen
FR	full resuscitation
G	group effect
GC-MS	gas chromatography-coupled mass spectrometry
GCS	Glasgow coma score
G*T	group*time interaction effect
H2S	hydrogen sulfide
HAT	histone acetylase
HBSS	Hank's balanced salt solution
HCA_2	hydroxyl-carboxylic acid receptor
HDAC	histone deacetylases
Hgb	hemoglobin
HIT	hibernation-induced 'trigger'
HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase
HMGCS2	3-hydroxymethyl-3-methylglutaryl-CoA synthase 2

HPβCD	hydroxypropyl-β-cyclodextrin
HR	heart rate
HSL	hormone sensitive lipase
IAP	intraabdominal pressure
IO	intraosseous
IQR	interquartile range
IV	intravenous
IVC	inferior vena cava
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
LDH	Lactate dehydrogenase
LR	lactated Ringer’s solution
$\Delta\psi m$	mitochondrial membrane potential
MAP	mean arterial pressure
PEG	polyethylene glycol 400
PVP	polyvinylpyrrolidone K12
M, MLT	melatonin
MCT	monocarboxylic acid transporter
MPAP	mean pulmonary arterial pressure
MPTP	mitochondrial permeability transition pore
MTD	maximum tolerated dose
Na_2S	disodium sulfide
NaHS	sodium hydrosulfide
OCR	oxygen consumption rate
OGD	oxygen glucose deprivation
OGD/R	oxygen glucose deprivation/reperfusion

$PaCO_2$	partial arterial pressure of carbon dioxide
PaO_2	partial arterial pressure of oxygen
PARP-1	poly [ADP-ribose] polymerase 1
PFK	phosphofructokinase 1
PR	post resuscitation
$PvCO_2$	partial pressure of venous carbon dioxide
R	limited resuscitation
RLU	relative luminescence unit
ROS	reactive oxygen species
RT	room temperature
S 45 min	end of shock period
SBP	systolic blood pressure
SCOT	succinyl-CoA:3-oxoacid-CoA transferase
SCN	suprachiasmatic nucleus
SCS	succinyl-CoA synthetase
SIRS	systemic inflammatory response syndrome
SvO_2	venous oxygen saturation
T	time effect
TCA cycle	cycle tricarboxylic acid cycle
Tg’	glass transition temperature of freeze-concentrate
TH	therapeutic hypothermia
UPLC-MS	ultrahigh-performance liquid chromatography-coupled mass spectrometry
UCP	uncoupling protein
UO	urine output
VCO_2	carbon dioxide production

VO_2	oxygen consumption
XRD	X-ray diffractometry

Chapter 1. General Introduction

Portions of this introduction were published in:

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Unlike many other causes of death, traumatic injury does not favor the old over the young. Injury is the leading cause of death in Americans ages 1-44, and the 4th leading cause of death overall (1). Worldwide, more than 5 million people die from injuries every year (2). Hemorrhagic shock, the state induced by severe blood loss, accounts for 30-40% of civilian trauma deaths (3). It is estimated that 10-20% of trauma deaths are preventable, with hemorrhagic shock being the leading cause of preventable death after injury (4).

Mechanisms of injury in hemorrhagic shock and ischemia/reperfusion injury

In hemorrhagic shock, a substantial drop in circulating blood volume and the resulting hemodynamic instability lead to deficient organ blood supply, known as ischemia. Decreased delivery of oxygen and nutrients results in a mismatch between tissue perfusion and cellular metabolic requirements (5, 6). In compensated shock, elevated sympathetic activity causes an increase in heart rate and vasoconstriction, preserving blood flow to critical organs (e.g. kidney, heart, brain) (7). If oxygen supply is too low to maintain aerobic respiration at adequate levels, cellular function is maintained via a partial switch to anaerobic respiration for ATP generation (6). If energy production drops further, ATP levels become insufficient to maintain cellular functions. In decompensated shock, ion transporters across the cellular membrane become dysfunctional, resulting in loss of membrane integrity, cellular swelling, and ultimately cell death (6). Exhausted stores of ATP, along with elevated adenosine nucleotide levels, prime the cells for ATP production (8). Increased anaerobic respiration and cellular dysfunction lead to buildup of lactate and raised generation of free radicals, resulting in acidosis, oxidative stress and ultimately organ injury and death (5, 6, 8).

Patients experiencing severe blood loss receive crystalloid resuscitation fluids and/or blood components to restore intravascular volume and stabilize hemodynamics. When

tissue perfusion is reestablished, rapid restoration of oxygen and energy substrates quickly reactivates cellular respiration and increases oxygen uptake and free radical generation (9). Reperfusion-induced oxidative stress causes cellular death and tissue damage, called reperfusion injury (7, 8). Reactive oxygen species can directly damage DNA, proteins and cell membranes (10). Oxidative stress also contributes to the opening of the mitochondrial permeability transition pore, resulting in mitochondrial dysfunction and further decreases in ATP levels (11, 12). Combined, these factors induce apoptotic and necrotic cell death (9).

Hemorrhagic shock is not an isolated event, but is often the result of traumatic injury. The direct mechanic insult of trauma adds to the adverse effects of acute blood loss. Pulmonary contusion, a common occurrence in major trauma, causes respiratory distress and hypoxemia (13). Pulmonary contusion can induce local and systemic immunodysfunction and increases the risk for infection and development of acute respiratory distress syndrome (14-18). Hepatic injury, which is often difficult to repair, can result in uncontrolled internal bleeding (19). Severe traumatic injury induces a local inflammatory response and activation of early immune response pathways (20-22). Combined, hemorrhagic shock and traumatic injury can lead to an overreaction of the innate immune response and the release of large amounts of pro-inflammatory mediators (7). This can then result in exacerbated systemic inflammation, known as the systemic inflammatory response syndrome (SIRS). SIRS is counteracted by a compensatory suppression of the immune response, called compensatory anti-inflammatory response (CARS) (7). The elevated activation of pro-inflammatory and anti-inflammatory immune responses predisposes patients to impaired organ function and ultimately multiple organ failure, a major contributor to late mortality after hemorrhagic shock (23, 24). Analysis of the circulating leukocyte transcriptome after severe trauma or burn injury showed that that these severe stresses induce a “genomic storm” which affects gene expression in over 80% of cellular functions (25). In this study, genes involved in the systemic inflammatory, innate immune and compensatory responses after severe trauma were upregulated simultaneously. Interestingly, severity of outcomes differed in the magnitude, but not the pattern of gene expression (25).

Current resuscitation approaches

Treatment of hemorrhagic shock is a comprehensive approach aimed to stop active hemorrhage and restore blood volume. Blood loss is controlled mechanically (e.g. tourniquets), surgically and/or with hemostatic agents (26). In addition, patients receive fluid resuscitation with the goal of restoring intravascular volume and increasing blood pressure and end organ perfusion (5). There is a myriad of resuscitation solutions available. Blood and blood components have the advantage of providing oxygen-carrying red blood cells and necessary blood clotting factors, but come with high cost, limited availability and the inherent risk of transfusion reactions and infections (27). Due to these challenges, resuscitation fluids containing salts (called crystalloids) or colloids (called colloidal solutions) are commonly used instead of or in addition to blood products.

The optimal fluid resuscitation strategy remains a topic of continuing debate (27). Studies have shown that massive fluid infusion and restoration of blood pressure to pre-hemorrhage levels are associated with increased blood loss, organ injury and mortality (28-30). The current standard of care is the rapid infusion of 1 – 2 l lactated Ringer's solution (a crystalloid) in the presence of symptoms of hemorrhagic shock (31). This is followed by hypotensive resuscitation (target SBP 80-90 mm Hg) to maintain tissue perfusion without increased bleeding or fluid overload (32).

The ideal resuscitation fluid should be efficacious, safe, cheap and stable during various storage conditions, without causing adverse effects. However, it has been increasingly recognized that currently used resuscitation fluids can exacerbate shock-induced injury (33). For example, infusion of racemic lactated Ringer's solution (LR) was associated with increased neutrophil activation in swine not just after hemorrhagic shock, but also in healthy animals (34). Furthermore, LR significantly increased neutrophil activity in human neutrophils *in vitro* (35).

Increased neutrophil activity was not observed after treatment with hypertonic saline (35, 36). Infusion of hypertonic solutions creates an osmotic surplus in the vasculature, which leads to a fluid shift from the extravascular space, thereby increasing intravascular volume (27, 37). Due to their low volume, hypertonic solutions provide a logistical advantage in extreme conditions (e.g. the battlefield). In 1999, the Institute of Medicine recommended the use of hypertonic saline as the initial resuscitation fluid after battle field injury (33). However, a recent meta-analysis did not find a significant mortality

difference between hypertonic saline and isotonic crystalloid (38). Furthermore, the analysis indicated that hypertonic saline significantly increased serum sodium levels when compared to isotonic saline. Today, the search for the ideal resuscitation fluid is still ongoing.

Hibernation-based treatment approaches for hemorrhagic shock

Hibernation-based treatment approaches have been of increasing interest for various biomedical applications, from treatments for stroke (39) to the induction of suspended animation during space travel (40). One hibernation-based approach, therapeutic hypothermia, is routinely used during cardiovascular and neurosurgery and to improve outcomes of cardiac arrest and neonatal asphyxia (41). Hibernation-based approaches are relevant to the treatment of hemorrhagic shock, as hibernators are innately protected from ischemia-induced injury and inflammation. Consequently, multiple hibernation-based treatment approaches have emerged for the treatment of hemorrhagic shock.

Figure 1.1. Hibernators. The arctic ground squirrel (*Spermophilus parryii*, left) and the hoary marmot (*Marmota caligata*, right) are both hibernators.

Torpor and hemorrhagic shock - similar but not the same

Hibernation is a transient state of inactivity and hypometabolism in endothermic (warm-blooded) animals. This adaptive phenomenon is known to occur in avian and mammalian species, including hummingbirds, bats, hedgehogs, marmots and ground squirrels (Figure 1.1) (42). Hibernation is characterized by hour- to week-long phases of fasting and inactivity known as torpor. Torpor is frequently interrupted by shorter interbout arousals, during which hibernators return to "normal" activity (42, 43). During these fast alternations between torpor and arousal, physiological changes resemble those during hemorrhagic shock and resuscitation. Similar to hemorrhagic shock, torpor is marked by global reductions in physiologic activity and metabolic rate, including decreased blood flow, heart rate, cardiac output, respiratory rate, and oxygen and nutrient supply and consumption (Figure 1.2 (42, 43)). These changes are succeeded by decreases in body temperature, which range from a few degrees below euthermic temperatures in bears to below freezing in the arctic ground squirrel (42, 43). Similar to reperfusion after hemorrhagic shock, arousal from torpor is characterized by rapid restoration of physiological parameters to pre-torpor levels. Heart rate and oxygen consumption increase, followed by a rise in body temperature (42-44).

Despite obvious similarities between hemorrhagic shock/resuscitation and torpor/arousal, important differences emerge (Table 1.1). Hibernation is an induced and highly regulated state, accompanied by seasonal changes in gene and protein expression and activation, which are associated with changes in nutrition and metabolism, coagulation, antioxidant defense and other factors (42, 45, 49). Body temperature and blood pressure decrease in both shock and hibernation; however during torpor, these parameters are maintained above critical levels (44). Torpor in seasonal hibernators is typically preceded by a period of preparation involving increased storage of body fat. This allows for a switch from carbohydrate to lipid metabolism, maintaining stable glucose levels and preventing muscle wasting during inactivity (50). In contrast, the early response to hemorrhagic shock and trauma is characterized by a hypercatabolic state with peripheral insulin resistance and hyperglycemia, along with increases in glycolysis, gluconeogenesis, glycogenolysis, lipolysis and fatty acid utilization, proteolysis and

increased levels of ketone bodies (51). Ultimately, depletion of glycogen stores can lead to hypoglycemia (52).

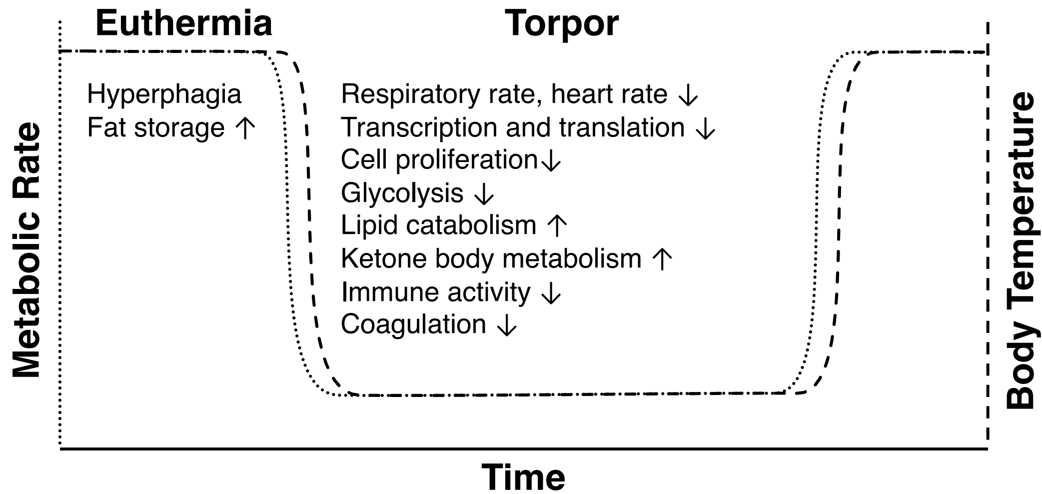

Figure 1.2. Physiological changes during torpor. Many hibernators undergo hyperphagia and increased fat storage in preparation for extended torpor. Torpor is marked by prominent decreases in respiratory, heart, and metabolic rate, which are succeeded by decreases in body temperatures to as low as -2.9° C (42, 43). Torpor is associated with global reductions in transcription and translation; however, steady-state levels of most proteins remain relatively constant (42). During torpor, induction of mitochondrial pyruvate dehydrogenase kinase 4 (PDK4) inactivates pyruvate dehydrogenase, suppressing glycolytic activity (42). Enhanced PPAR-γ and PGC-1α expression is associated with increased lipid catabolism (45, 46). Elevated levels of ketone bodies suggest that these fuel sources are utilized at increased rates during torpor (47, 48). Lastly, torpor is associated with reversible immunosuppression and increased clotting times (42, 45).

Table 1.1. Comparison of physiologic functions during hemorrhagic shock and torpor

	Hemorrhagic Shock	**Torpor**
Timeline	Sudden & unexpected	Preparation with hyperphagia and fat storing
Body temperature	Spontaneous hypothermia is common	Near ambient temperature, thermoregulation is maintained to keep temperature above critical levels
Hemodynamics	Tachycardia, hypotension	Bradycardia, moderate but stable hypotension
Oxygen consumption	Delivery < consumption	↓ Delivery ~ ↓ consumption
Metabolism	Switch from aerobic to anaerobic metabolism, hypercatabolic state	Greatly reduced metabolic rate, switch from carbohydrate to lipid metabolism
Cellular function	Ischemia depletes ATP, reperfusion increases ROS production	Maintained
Coagulation	Coagulopathy	Reversible hypocoagulation
Immune system	SIRS, CARS	Leukocytopenia and immunosuppression during torpor, reversed upon arousal

Hibernators show improved resistance to ischemia/reperfusion injury and hemorrhagic shock

In contrast to patients suffering from hemorrhagic shock, hibernating mammals undergo torpor and arousal without detriment to their health (53). This indicates that these animals are uniquely equipped to deal with abrupt changes in tissue perfusion and metabolism. Indeed, hibernators exhibit superior tolerance to hypoxia and ischemia/reperfusion injury when compared to non-hibernators (48, 54-58).

What lies behind this protective effect? One study detected differences in gene expression patterns in summer and interbout arousal ground squirrels, which remained when the animals were exposed to ischemia/reperfusion injury (49). Increased availability and use of non-glucose energy sources during hibernation provides flexibility and may stabilize metabolism after ischemia/reperfusion insult (59). For example, hibernating ground squirrels experience significantly increased serum levels and utilization of ketone bodies, which have been associated with improved hypoxic tolerance (47, 48, 60). While some research suggests that protection from ischemia/reperfusion injury is linked to winter or the torpid state (56-58), other studies observed increased tolerance also in active hibernators during the summer (54, 55). Hence, the protective mechanisms in hibernators may be available throughout the year.

Improved ischemia/reperfusion tolerance in hibernating species extends to instances of hemorrhagic shock. ^{1}H-NMR metabolomics analysis suggested lower reliance on anaerobic metabolism, and decreased glucose turnover and organ dysfunction after hemorrhagic shock in both interbout arousal and summer euthermic ground squirrels when compared to rats (59). In a hemorrhagic shock and resuscitation model, all of the tested rats expired within 18 hours after reperfusion, while both euthermic and interbout arousal arctic ground squirrels survived for 72 hours (54). Arctic ground squirrels also experienced decreased kidney injury and systemic inflammation, along with higher pH and base excess, suggesting better metabolic protection from acid-base imbalance (54). Utilizing the adaptive mechanisms that protect hibernators from shock-induced injury and inflammation may help alleviate the detrimental effects of hemorrhagic shock in non-hibernating species. Indeed, various hibernation-based treatment approaches have shown promising effects in hemorrhagic shock (Table 1.2).

HIT and DADLE

HIT and DADLE induce hibernation-like effects

Dawe and Spurrier were the first to show that intravenous injection of whole blood or blood components from torpid hibernators induced hibernation in euthermic, active summer ground squirrels (61). The authors concluded that an (unknown) hibernation-induced 'trigger' (HIT) for natural mammalian hibernation could be transferred via transfusion. These effects are not limited to hibernators. Treatment with HIT transiently decreased body temperature and oxygen consumption in rodents (62) and caused transient hypophagia, bradycardia, hypothermia and lethargy in primates (63). In summer-active ground squirrels, treatment with the delta opioid receptor agonist D-Ala^2-Leu^5-enkephalin (DADLE) induced hibernation comparable to that after infusion of HIT (64). Both HIT and DADLE likely exert their effects through activation of the endogenous delta-opioid system. DADLE activates delta opioid receptors directly, and may have additional non-opioid-effects (39, 65). In contrast, HIT does not seem to be an opioid-receptor agonist itself, but likely induces its effects after conversion to an active form, or by inducing the release of endogenous opioids (65).

HIT and DADLE improve outcomes after ischemia/reperfusion and hemorrhagic shock

HIT and DADLE significantly improved organ function and survival in various animal organ preservation models and increased ischemia/reperfusion tolerance in isolated heart, skeletal muscle and jejunum (65, 66). HIT or DADLE pretreatment, but not acute administration significantly improved function and ultrastructural preservation in isolated rabbit hearts exposed to cardioplegia (67). *In vivo*, DADLE significantly improved markers of lipid peroxidation and hepatocyte injury in a rat hepatic ischemia/reperfusion model (68). In rats exposed to middle cerebral artery occlusion, DADLE significantly decreased cerebral infarction volume, cellular apoptosis, oxidative stress and inflammation, increased antioxidant enzymes and attenuated behavioral impairments (39, 69).

Despite promising results in ischemia/reperfusion models, data on the effects of DADLE in hemorrhagic shock are limited. DADLE infusion prior to blood withdrawal significantly improved survival and decreased lactate levels in rats (70). In contrast, there was no significant difference in survival, neurological status or histological damage score

in a rat model of hemorrhagic shock and emergency preservation and resuscitation model where DADLE was added during cold aortic flush and resuscitation (71). Injection into the hypothalamus at the end of hemorrhage did not affect mean arterial pressure or heart rate in hypovolemic rats (72). The variable results in preclinical hemorrhagic shock models suggest that the timing of DADLE administration affects its efficacy. While DADLE treatment is beneficial when given as pretreatment (39, 67, 68, 70), treatment onset during ischemia or reperfusion is generally not associated with beneficial effects (67, 71, 72). Only one study found that DADLE was protective when injected during cerebral ischemia (69). The time-dependent effects of DADLE raise doubts that this treatment can be effectively applied in clinical practice.

Hydrogen sulfide

Hydrogen sulfide (H_2S) has become appreciated for its function as a gasotransmitter in the vasculature, the nervous system and in inflammation (73). H_2S may be involved in ischemia/reperfusion protection during the transition from torpor to arousal (74). Gaseous H_2S can be administered via inhalation; however, H_2S inhalation exerts pulmonary toxicity (75). Alternatively, the inorganic sulfides Na_2S and NaHS act as donors that release H_2S into solutions which can be administered intravenously. At increased doses, H_2S is toxic due to its inhibition of cytochrome c oxidase, thereby decreasing mitochondrial respiration (73).

Hydrogen sulfide decreases metabolic rate in small animals

About a decade ago, researchers first reported that H_2S induces a “suspended animation-like state” in small animals (76). Mice exposed to H_2S gas experienced substantial decreases in metabolic rate, body temperature, cardiac output, heart and respiratory rate and physical activity (76-78) and returned to normal physiological function without obvious impairments once moved back to normal air (76). The metabolic effects of H_2S are less straight forward in larger rodents and other mammals. Studies found contradicting results in rats (79, 80), and H_2S did not decrease body temperature or metabolic rate in healthy sedated sheep or piglets (75, 79, 81). In humans, inhalation of 10 ppm H_2S during exercise significantly decreased oxygen consumption and increased blood lactate, with no obvious effects on CO_2 production or heart rate (82).

Responses to H_2S treatment may vary with species differences in baseline metabolic rate or temperature regulation, or may have been masked by sedation in larger animals (66).

Hydrogen sulfide improves outcomes of ischemia/reperfusion injury and hemorrhagic shock

Pretreatment with H_2S greatly extended survival without inducing behavioral defects in mice exposed to oxygen tensions as low as 3% (77). H_2S-treatment is protective during organ preservation and ischemia/reperfusion injury in various organs, including the heart, liver, kidney, brain, stomach, intestine and the lung (73). Treatment with H_2S gas or sulfide solution during hemorrhage stabilized CO_2 production, a surrogate marker of metabolism, significantly improved survival, and attenuated shock-induced decreases in $PaCO_2$, HCO_3^-, and base excess and increases in blood potassium and lactate levels in rats (80). In contrast, H_2S infusion during resuscitation did not result in significant differences in mean arterial pressure, markers of organ injury, or survival in a porcine hemorrhagic shock model (83).

Intraperitoneal or intravenous injection of NaHS improved survival, mean arterial pressure, cardiac and vascular function, cerebral blood flow, metabolic acidosis, inflammation, oxidative stress and markers of organ injury and apoptosis in rat hemorrhagic shock (84-88). In a porcine model of hemorrhagic shock and truncal ischemia, Na_2S infusion during resuscitation significantly decreased organ damage and lowered epinephrine requirements for blood pressure control (89). Continuous Na_2S infusion significantly improved survival in pigs exposed to hemorrhagic shock independent of whether this treatment was started before, during or after blood loss; however, shock-induced organ injury, inflammation, and decreases in base excess were only attenuated when Na_2S infusion was initiated at the onset of hemorrhage (90). Combined, the described studies suggest that H_2S and sulfide solutions improve outcomes of hemorrhagic shock in both rats and pigs, and that efficacy may vary depending on the timing of treatment administration.

Effectiveness of H_2S may be diminished by its immune-modulatory effects. Hemorrhagic shock increased plasma and tissue levels of H_2S in rats (91, 92). Decreasing H_2S levels lowered inflammation in these models, suggesting that H_2S is pro-inflammatory (91, 92). In humans, shock-survivors showed lower total plasma sulfide

concentrations than non-survivors (93). However, other studies reported anti-inflammatory effects of H_2S in hemorrhagic shock (85) and ischemia/reperfusion injury (73). The immune-modulatory profile of H_2S remains to be completely characterized, and pro-inflammatory effects of H_2S may increase the risk of injury-related inflammation and sepsis.

The variability in the outcomes of treatment with H_2S or sulfide salts in healthy animals and those exposed to hemorrhagic shock underlines potential difficulties for the translation of this treatment into clinical use. Pulmonary and general toxicity limit the dose that can be safely administered. Lower metabolic rates and differences in thermoregulation may render the effects of H_2S less prominent in larger animals. Time-dependent treatment effects may result in decreased efficacy when administration is delayed. While treatment before or at the onset of reperfusion generally exerts beneficial effects (80, 84-90), administration during resuscitation was not effective (87). Lastly, H_2S is quickly lost from solution, making it difficult to administer precise doses. Slow-release H_2S donors may allow for more accurate H_2S dosing while extending treatment duration (94). However, these substances have not been tested in hemorrhagic shock models.

Ghrelin

Ghrelin is a peptide hormone that is synthesized in the stomach upon food deprivation. It has been suggested that Ghrelin plays a role in food intake, body mass and energy balance in hibernators (95). Ghrelin administration decreased body temperature during fasting-induced torpor in mice, but not in non-fasting rodents or healthy volunteers (96, 97). In a rat model, ghrelin administration 45 minutes after traumatic brain injury and uncontrolled hemorrhage significantly increased survival, attenuated brain injury and inflammation, and facilitated cognitive recovery (98). Despite these interesting initial results, ghrelin has not been tested in larger animal models, and it is unknown whether the treatment could be translated to clinical use.

Adenosine, lidocaine and magnesium

During torpor, cardiac activity is substantially decreased; however, the resting membrane potential in the heart is maintained (99). This observation led to the

development of a combination of adenosine, lidocaine and magnesium (ALM), which arrests the heart while maintaining the membrane potential near its polarized resting state (polarized cardioplegia (100)). ALM exerts protective effects in animal models of cardiac arrest (100). While the mechanism of action of ALM is not exactly known, it is likely a combination of the individual effects of each of the solution components. These include the reduction of oxygen demand through antiadrenergic effects, a decrease in ischemia/reperfusion injury by lowering cellular calcium entry and scavenging free radicals, a decrease in neutrophil activation and inflammation, decreased arrhythmias, and improved cardiac function and coagulation (100).

In rat hemorrhagic shock, ALM treatment significantly improved survival, decreased ventricular arrhythmias and hypocoagulopathy, and attenuated shock-induced decreases in mean arterial pressure (101, 102). A recent study further showed that ALM significantly improved post-shock microvascular glycocalyx integrity (103). In a pig hemorrhagic shock model, animals received ALM at the onset of hypotensive resuscitation, followed by a bolus of adenosine and lidocaine (AL) at the reinfusion of shed blood. ALM/AL treatment increased mean arterial pressure and cardiac index, decreased crystalloid infusion requirements for post-shock blood pressure control, attenuated shock-induced lactic acidosis, increased base excess and HCO_3^-, raised urine output and improved renal and cardiac function (104, 105). The beneficial results obtained in rat hemorrhagic shock models translate into pigs, although the effect on survival remains to be determined. ALM improves outcomes when administered at the onset of resuscitation, making it effective in a clinically relevant treatment regimen.

Hypothermia

Shock-induced exhaustion of ATP stores, which is exacerbated by shivering, diminishes heat production (106, 107). Post-shock spontaneous hypothermia is associated with increased acidosis and hypocoagulopathy, resulting in a “vicious cycle” that can lead to death if left untreated (108). It correlates with injury severity and significantly increases the risk for development of multiple organ dysfunction syndrome (109, 110). Some, but not all studies found that spontaneous hypothermia independently increased mortality in severely injured patients (109-111). Due to the association of post-injury

hypothermia with worsened outcomes, current trauma care guidelines call for the prevention and reversal of decreases in body temperature.

Therapeutic hypothermia improves outcomes of ischemia/reperfusion injury and hemorrhagic shock

In therapeutic hypothermia, body temperature is tightly controlled. Shivering is suppressed while body temperature is actively decreased by surface cooling or infusion of cold fluids. In preclinical studies, therapeutic hypothermia is commonly classified as mild (33-36 ºC), moderate (28-32 ºC), deep (16-27 ºC), profound (6-15 ºC), or ultra-profound (<5 ºC) (106).

Therapeutic hypothermia is beneficial in animal models of ischemia/reperfusion injury and hemorrhagic shock. In rats exposed to severe hemorrhage, induction of mild or moderate hypothermia significantly increased survival (112, 113). Hypothermia further increased mean arterial pressure, decreased heart rate, lowered fluid requirements, and attenuated shock-induced organ injury and increases in lactate levels, vascular permeability, markers of inflammation, and oxidative stress (112-115). In porcine hemorrhagic shock, mild hypothermia significantly improved survival and decreased lactate levels and markers of organ dysfunction (116, 117).

Emergency preservation and resuscitation (EPR) is an experimental procedure that induces suspended animation via profound hypothermia to improve outcomes after exsanguination cardiac arrest. Hypothermia is rapidly induced via aortic flush with intravenous fluids, followed by circulatory arrest, reparative surgery and rewarming and resuscitation with cardiopulmonary bypass (118). EPR has been extensively studied in preclinical trauma models, and results have recently been evaluated in a systematic review (118). In rats, EPR significantly increased survival and decreased neurological impairment and kidney injury after 60 minutes, but not 75 minutes of shock (119). EPR allowed for survival of up to 120 minutes of circulatory arrest with only mild neurological deficit in dogs (120). In pigs subjected to 120 minutes of circulatory arrest followed by EPR, 50% of animals survived and most showed no neurological or organ dysfunction (110). EPR outcomes might be further improved by optimization of the cooling fluids. Addition of glucose and oxygen to the flush solution allowed for survival of three hours of circulatory arrest without severe neurological impairment in dogs (121). The efficacy of therapeutic hypothermia and EPR is affected by various factors, including

etiology, depth, duration, the rate of induction and rewarming, severity of injury, method of cooling and the species evaluated (106, 118). Because of the suggested improvements in outcomes, therapeutic hypothermia may be a means to achieve survival of hemorrhagic shock and exsanguination cardiac arrest.

The beneficial effects of therapeutic hypothermia are likely a result of ischemia protection, inhibition of post-injury inflammation and activation of pro-survival pathways (Figure 1.3). Hypothermia decreases metabolic rate and oxygen consumption (106, 117). By increasing mean arterial pressure while lowering heart rate, hypothermia leads to decreased cardiac energy consumption, while cardiac output is maintained (113, 122). Combined, these effects preserve aerobic respiration and cellular ATP levels and decrease oxidative damage during ischemia and reperfusion (114, 123-125). Hypothermia blunts the shock-induced immune response, thereby decreasing inflammation and organ injury (125-128). Furthermore, therapeutic hypothermia induces changes in gene expression, resulting in increased levels of survival proteins like bcl-2 and β-catenin, while pro-apoptotic proteins are decreased (106).

Multiple hurdles remain until therapeutic hypothermia can be applied in shock patients. Protocols utilized in lab settings may not be feasible in the clinic, especially during the pre-hospital phase. Target temperature may vary with shock and injury severity; however, estimating the amount of lost blood can be difficult. Further research is necessary to examine the efficacy of therapeutic hypothermia if applied once spontaneous hypothermia has begun. The timing, rate of cooling and rewarming, as well as duration of hypothermia need to be optimized to generate therapeutic benefits without induction of hypothermia-induced adverse effects like arrhythmias, changes in serum electrolytes, insulin resistance, reversible hypocoagulopathy and increased risk for infection (106, 107). Lastly, slowed drug metabolism during hypothermia increases the risk of toxicity (107). It remains to be tested whether the promising preclinical results can be translated into bedside care. A clinical trial evaluating the efficacy of emergency preservation and resuscitation after exsanguination cardiac arrest from trauma is currently underway (129).

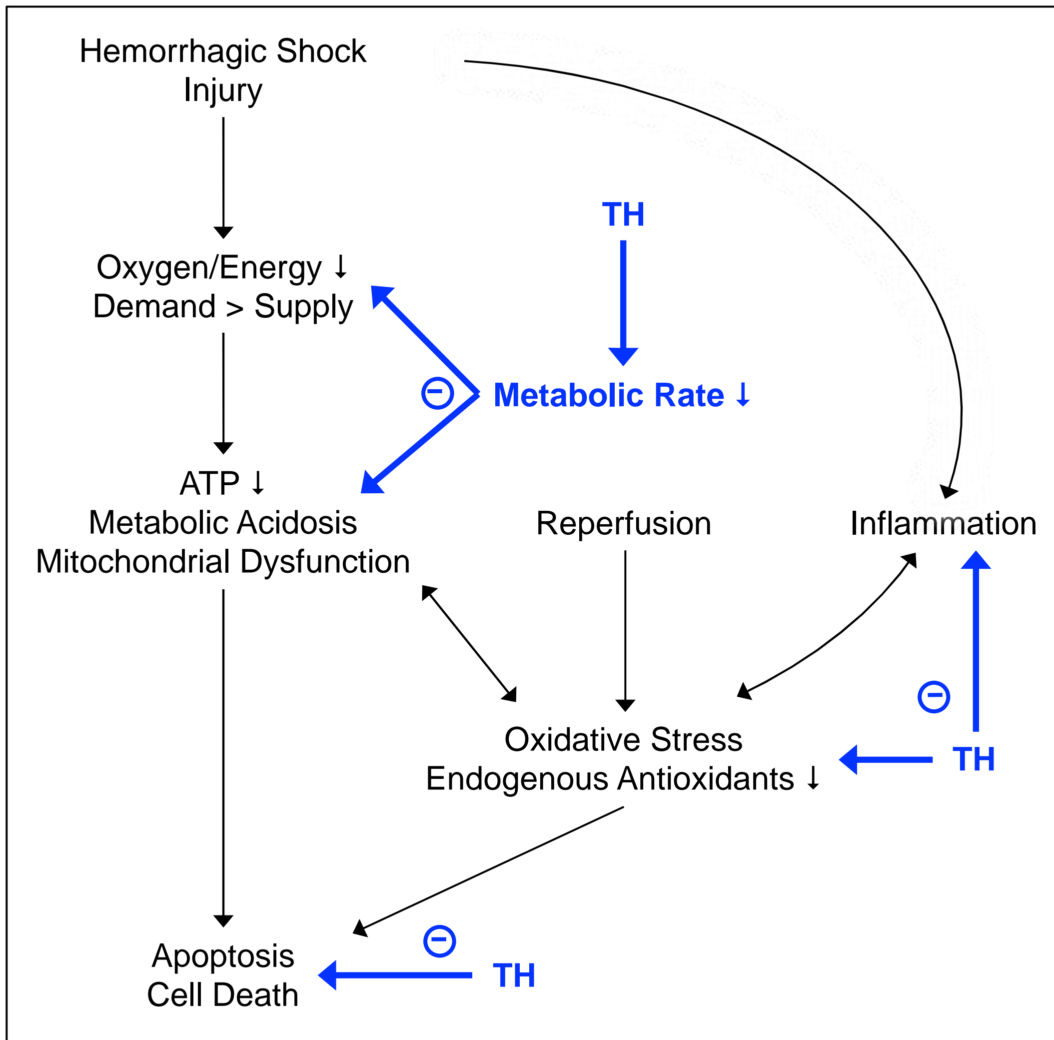

Figure 1.3. Effects of therapeutic hypothermia in hemorrhagic shock. By decreasing the metabolic rate, therapeutic hypothermia (TH) lowers oxygen and energy demands, thereby attenuating shock-induced losses in ATP and consecutive oxidative stress and cellular impairment (106, 114, 117, 123-125). TH has also been shown to blunt the shock-induced inflammatory response (125-128). TH prevents apoptosis by increasing expression of the pro-survival proteins beta-catenin and bcl-2 through the Akt signalling pathway, while attenuating release of the pro-apoptotic protein bax and inhibiting caspase-dependent apoptosis pathways (106).

D-β-hydroxybutyrate and melatonin

D-β-hydroxybutyrate

Treatment with a combination of D-β-hydroxybutyrate (BHB) and melatonin (M) increases survival in preclinical hemorrhagic shock models. BHB is a ketone body, which serves as a glucose alternative fuel source in various extrahepatic tissues, including the heart, the brain, the kidney and in skeletal muscle (47, 130-132). Blood ketone body concentrations increase with decreased food intake, from below 0.1 mM postprandial to 1-2 mM after two days of fasting (133, 134), to 6-8mM after prolonged starvation (135, 136). A low carbohydrate diet or intense exercise also increases systemic ketone levels to 1- >2 mM (137, 138). Increased ketone levels inhibit glucose utilization, and ketone bodies can substitute glucose as an energy source in many tissues, allowing substrate oxidation and ATP production to continue when glucose is sparse (133, 139). Furthermore, BHB metabolism is independent of insulin, and ketone bodies provide energetic flexibility in conditions of blunted insulin activity (140, 141).

Ketone production (ketogenesis) and ketone body utilization (ketolysis) are illustrated in Figure 1.4. Ketogenesis mainly occurs in the liver (142), although other tissues may generate small amounts of ketone bodies (143, 144). The rate of ketogenesis is governed mainly by hepatic production of acetyl-CoA from beta-oxidation of free fatty acids (145). In the liver mitochondria, acetyl-CoA is converted first to acetoacetyl-CoA and then irreversibly to HMG-CoA, which is cleaved to acetoacetate. D-BHB dehydrogenase converts acetoacetate to BHB in a reversible reaction. BHB and acetoacetate exist in equilibrium, which is driven by the NaD^+ to NADH redox ratio (145). BHB and acetoacetate exit the liver via monocarboxylic acid transporters 1, 2 and 7 (146, 147). BHB and acetoacetate enter tissues via monocarboxylic acid transporters 1 and 2 (47, 147, 148), where BHB is oxidized to acetoacetate with the reduction of NAD^+ to NADH. Acetoacetate is converted to acetoacetyl-CoA by succinyl-CoA:3-oxoacid-CoA transferase (SCOT), which also catalyzes the conversion of succinyl-CoA to succinate. SCOT is not found in the liver, which means that while the liver is the main producer of ketone bodies, it does not utilize them itself (149). Acetoacetyl-CoA is cleaved to two acetyl-CoA molecules which enter the tricarboxylic acid (TCA) cycle. The TCA cycle generates NADH and $FADH_2$, reducing elements used by the electron transport chain for ATP production via oxidative phosphorylation.

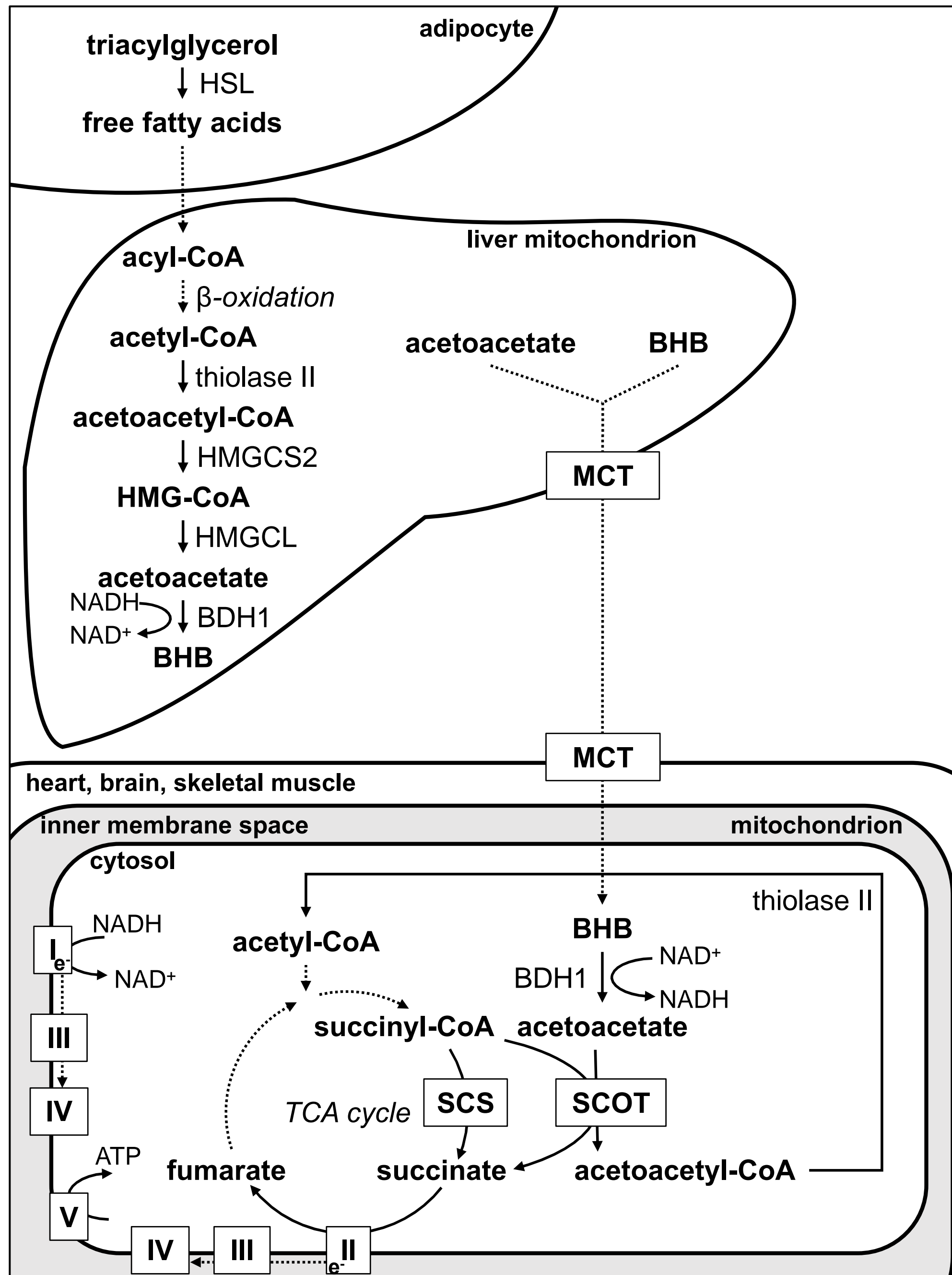

Figure 1.4. Ketogenesis and Ketolysis. I-V - complexes 1-5 of the electron transport chain (I – NADH dehydrogenase, II – succinate dehydrogenase, III – ubiquinol cytochrome c oxidoreductase, IV – cytochrome c oxidase, V – ATP synthase), BHB – D-β-hydroxybutyrate, BDH1 – BHB-dehydrogenase, HMGCL – 3-hydroxymethyl-3-methylglutaryl-CoA Lyase, HMGCS2 – 3-hydroxymethyl-3-methylglutaryl-CoA synthase 2, HSL – hormone sensitive lipase, MCT – monocarboxylic acid transporter, SCOT – succinyl-CoA:3-oxoacid-CoA transferase, SCS – succinyl-CoA synthetase, TCA cycle – tricarboxylic acid cycle.

BHB administration exerts beneficial effects in *in vitro* and *in vivo* hypoxia (60, 150). Endogenous BHB levels increased after ischemia (151), and BHB treatment improved energy metabolism, attenuated cerebral edema and decreased cardiac apoptosis and infarct size after local ischemia/reperfusion (60, 152). Resuscitation with LR in which lactate was replaced by BHB prevented decreases in base excess and attenuated increases in serum lactate and markers of inflammation and apoptosis in preclinical hemorrhagic shock models (153, 154).

Melatonin

Melatonin is synthetized from serotonin in a two-step process (Figure 1.5) (155). The pineal gland is the main producer (155), although other tissues, including the skin, retina, gut, platelets and bone marrow are also capable of producing melatonin (156-159). Melatonin is secreted into the circulation, from where it is widely distributed throughout the body. Melatonin serum levels closely follow pineal activity (160-162), which is driven by an endogenous clock in the suprachiasmic nucleus in the hypothalamus (163). Melatonin production follows the daily light/dark cycle (164), with highest serum levels at night (165, 166). Ultimately, most of the endogenously produced melatonin is hydroxylated to 6-hydroxymelatonin in the liver (167), which is conjugated with sulfate or glucuronide and excreted in urine (168).

Melatonin has various functions. It acts as a synchronizer and stabilizer of circadian rhythm (169, 170), and participates in regulation of the sleep-wake cycle (171). Furthermore, melatonin is involved in regulation of the annual reproductive cycle and other seasonal changes (172, 173). The effects of melatonin are exerted both through receptor binding and directly through its free radical scavenging properties. Two G-protein-coupled melatonin membrane receptors have been identified in mammals, MT1 and MT2 (174, 175), which are expressed throughout the body virtually every organ (176). Melatonin also binds to the orphan nuclear hormone receptor family RZR/ROR, which may relay some of its immune-modulatory effects (177).

While it has long been known that melatonin affects the immune system, its immune-modulatory effects are not straight forward. Melatonin administration increases thymus and spleen weight in rodents, suggesting an immune-stimulating effect (178-180). Furthermore, melatonin increases proliferation and activity of immune cells (181-184)

and induces cytokine production (185, 186). In contrast, other studies have shown anti-inflammatory melatonin effects (187-189). For example, melatonin reduced post-shock levels of pro-inflammatory cytokines, attenuated lipid peroxidation, prevented shock-induced circulatory failure and increased survival in animal models of septic shock (190-192). The differences in the observed effects may be due to differences in treatment regimens, as melatonin effects are dose-dependent (193, 194), vary with the time and duration of administration (195), and may be masked by changes in endogenous levels of melatonin (195).

Melatonin plays a prominent role in the control of oxidative damage. It not only increases expression of antioxidant enzymes (196-199), but also is a potent free radical scavenger (200-202). Melatonin reaction with reactive oxygen species produces cyclic 3-hydroxymelatonin and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), which themselves act as free radical scavengers (203-206). Some antioxidant functions of melatonin are mediated through receptor binding (207).

Melatonin plays a role in the regulation of mitochondrial homeostasis. Mitochondria not only produce the majority of the body's ATP, but are also a major source for reactive oxygen species (208). In mitochondria, electrons are shunted through complexes of the electron transport chain. This transfer is coupled with the transport of protons across the inner mitochondrial membrane, generating an electrochemical gradient that drives the synthesis of ATP (209, 210). During this process, low levels of superoxide radicals (O_2^-) are produced, which are subsequently converted into H_2O_2 and OH^- (211, 212). While low levels of reactive oxygen species are physiologic, increased production of free radicals is damaging to the electron transport chain (213). The resulting mitochondrial dysfunction further increases reactive oxygen production, feeding into a vicious cycle that can produce large amounts of reactive oxygen species and ultimately result in cell death (214). As described above, melatonin provides protection from oxidative damage through its radical scavenging and antioxidant effects. Consequently, melatonin increases activity of complexes of the electron transport, thereby maintaining mitochondrial function in the context of oxidative damage (215-219).

Countless studies have shown protective effects of melatonin in various organs, including in the brain, the heart, the liver, and the kidney (220-224). The beneficial

effects of melatonin in hypoxia and ischemia/reperfusion injury are well documented in both animal models and in patients (225). Melatonin treatment decreased resuscitation fluid requirements, prevented shock-induced decreases in mean arterial pressure and ameliorated shock-induced tachycardia and markers of apoptosis, oxidative stress and organ injury in rat and mouse models of hemorrhagic shock (226-229). In mice exposed to shock followed by septic challenge, melatonin treatment significantly increased survival (230).

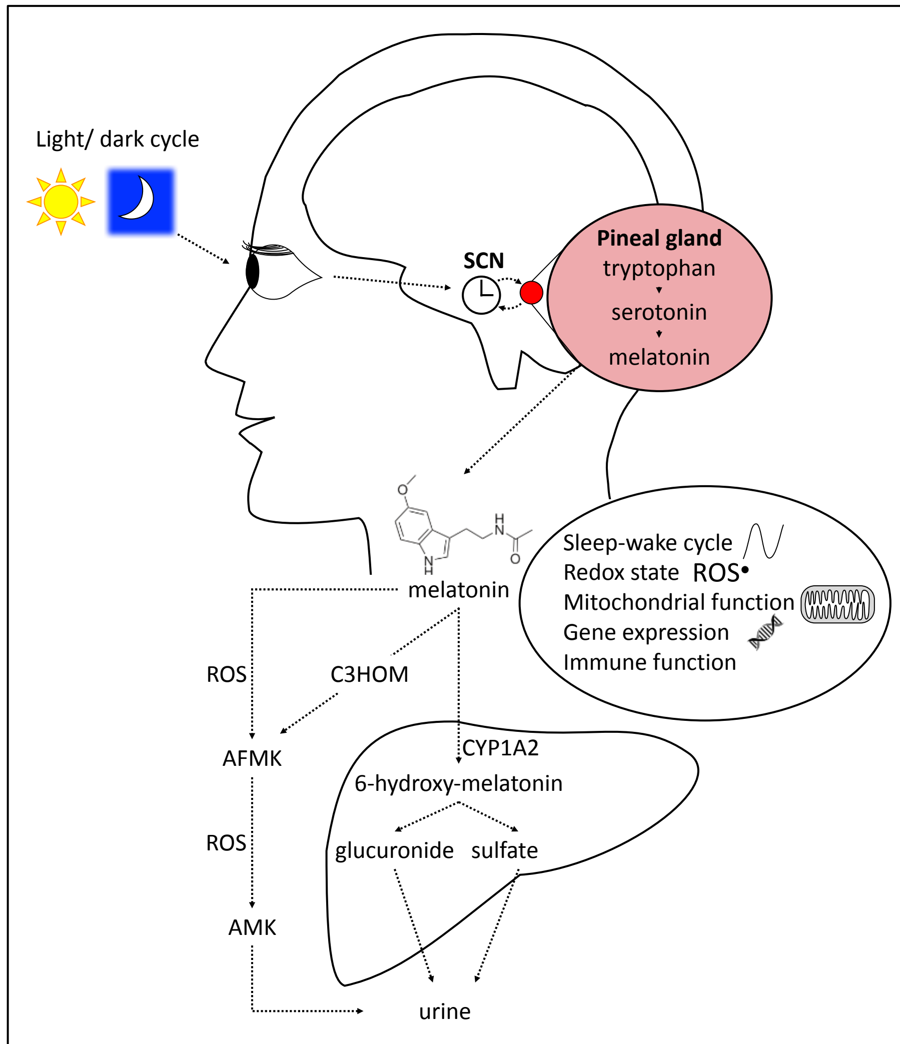

Figure 1.5. Production, Metabolism and Actions of Melatonin. AFMK – N1-acetyl-N2-formyl-5-methoxykynurenamine; AMK - N1-acetyl-5-methoxykynuramine; C3HOM – cyclic 3-hydroxymelatonin; CYP1A2 – cytochrome P450 1A2; ROS – reactive oxygen species; SCN – suprachiasmatic nucleus

D-β-hydroxybutyrate and melatonin

A small-volume infusion solution containing 4 M D-β-hydroxybutyrate and 43 mM melatonin (BHB/M) was developed after the observation that torpor and arousal are accompanied by increased levels of BHB and melatonin, respectively (231). BHB/M infusion starting during hemorrhagic shock significantly increased survival in both rat and pig hemorrhagic shock (231-233). Furthermore, BHB/M attenuates shock-induced microvascular endothelial glycocalyx degradation, which is increasingly recognized as a valuable target for hemorrhagic shock resuscitation (103). BHB/M is effective when treatment is initiated during blood loss, making it a promising first-line approach. However, whether efficacy is retained with delayed treatment onset remains to be tested.

Administration of BHB or melatonin alone is beneficial in hypoxia and ischemia/reperfusion injury (60, 150, 152, 225). In hemorrhagic shock, resuscitation with LR in which lactate was replaced by BHB prevented decreases in base excess and attenuated increases in serum lactate and markers of inflammation and apoptosis (153, 154). Melatonin treatment lowered resuscitation fluid requirements, prevented shock-induced decreases in mean arterial pressure and ameliorated shock-induced tachycardia and markers of apoptosis, oxidative stress and organ injury in rat and mouse hemorrhagic shock models (226-229). In mice exposed to shock followed by septic challenge, melatonin treatment significantly increased survival (230).

As described, BHB increases metabolic flexibility when glucose is limited or glucose utilization is impaired. BHB-receptor binding has been associated with reduces in energy expenditure via GPR41 antagonism (234) and neuroprotective effects in stroke via hydroxylcarboxylic acid receptor 2 (235), respectively. However, whether these receptors contribute to the effects of BHB in hemorrhagic shock remains to be determined.

Hemorrhagic shock induces an imbalance in the activity of histone acetylases (HATs) and histone deacetylases (HDACs), resulting in distinct changes in gene expression patterns (236, 237). HDAC inhibition increased survival in rat and swine models of hemorrhagic shock (238). Studies have shown that BHB inhibits class 1 HDAC *in vitro* and *in vivo* (239), suggesting that it may exert its beneficial effects through the upregulation of pro-survival pathways. BHB-induced changes in gene expression and metabolic state are closely linked. On the one hand, BHB metabolism provides NADH and $FADH_2$, reducing equivalents for aerobic ATP production. On the other hand,

changes in NADH and Acetyl-CoA, an intermediate of BHB metabolism, affect activities of HAT and HDAC (239).

During ischemia, the cellular antioxidant defense decreases, rendering cells prone to oxidative damage upon reperfusion (240). Countless studies have shown that melatonin not only acts as an antioxidant itself, but enhances the activity of endogenous antioxidants and antioxidant enzymes (225). Melatonin-induced decreases in post-shock markers of oxidative stress were partially reversed by treatment with melatonin receptor antagonists (227), suggesting that melatonin exerts beneficial effects through its inherent and receptor-mediated antioxidant activity.

The presented studies indicate that BHB and melatonin act synergistically in hemorrhagic shock (231, 232). Improved metabolic state and increased antioxidant defenses likely result in improved mitochondrial function and decreased oxidative stress after hemorrhagic shock. This effect may be enhanced by modulation of gene-expression and the inflammatory response.

Dimethyl sulfoxide

Because melatonin has limited solubility in water, the standard BHB/M formulation (4M BHB, 43mM melatonin) contains 20% of the dimethyl sulfoxide (DMSO). DMSO is an aprotic compound that is commonly used as a solubilizer in biological *in vitro* and *in vivo* studies (241). DMSO is approved for use in topical formulations (242), for the delivery of medical polymers in sustained release implants or medical devices (Viadur ®, Onyx™), and for intravesical administration as a treatment of interstitial cystitis (RIMSO-50®, reviewed in (243). The food and drug administration (FDA) inactive ingredient database lists DMSO as part of a powder for injection suspension at an unknown amount (244).

DMSO has acute low toxicity (intravenous LD50 in rats is > 5 g/kg, (245)); however, adverse effects after DMSO administration have been reported. The most common adverse effect of DMSO is nausea and vomiting due to garlic-oyster-like breath after pulmonary excretion of its metabolite dimethyl sulfide (246). DMSO induces concentration-dependent hemolysis, which is not associated with kidney injury (247). DMSO is commonly used as a cryoprotectant in hemopoietic stem cell and platelet infusion preparations at concentrations between 5 and 10% (248). Infusion of

hematopoietic stem cells can be accompanied by mild to severe or fatal adverse reactions, including nausea and vomiting, headaches, hemolysis, cardiac arrhythmias, encephalopathy, seizures, respiratory depression and renal failure (246-252). In the literature, these adverse effects were often blamed on DMSO (251). However, other factors can be associated with the occurrence of these events, including cell clumping, the amount of cell debris or granulocytes in the infusion, as well as patient-specific factors (248, 249, 253). Noteworthy, numerous studies did not report significant adverse effects after infusion of cryopreserved platelets containing up to 10% DMSO (254).

In contrast to its potentially unfavorable effects, DMSO was shown to be beneficial in the treatment of cardiac and central nervous pathologies as well as hemorrhagic shock (255). DMSO treatment decreased intracranial pressure and increased cerebral perfusion pressure in patients with closed head traumatic brain injury (256, 257). In a clinical pilot study, patients suffering from ischemic stroke were treated with repeated intravenous infusions of a combination of fructose 1,6-diphosphate (FDP) and DMSO, which were well tolerated (258). 3 months after treatment, 67% of patients receiving DMSO-FDP experienced improved neurological status, versus 20% of those receiving standard treatment. A recent publication evaluated the effects of DMSO on liver, renal and intestinal activation of the pro-inflammatory transcription factor NFκB and the heat shock protein Hsp70 in a rat model of hemorrhagic shock (Bini, 1048, 2008). In this study, intraperitoneal DMSO injection increased Hsp70 expression and attenuated shock-induced NFκB activation when compared to treatment with normal saline (259).

Table 1.2. Effects of different hibernation-based treatments in preclinical hemorrhagic shock models

Treatment	Proposed Mechanism	Beneficial Effects	Disadvantages
D-Ala[2]-Leu[5]-enkephalin (DADLE)	Delta opioid receptor agonist	↑survival, ↓ lactate (rat) (70)	• Not effective in all studies (rat) (71, 72) Not tested in larger animals
H_2S, NaHS, Na_2S	Decreases metabolic rate and body temperature	• H_2S ↑ survival, ↓ VCO_2, ↓lactic acidosis (rat) (80) • NaHS ↑ survival, ↑ MAP, ↑ cerebral blood flow, ↑ cardiac function, ↓ metabolic acidosis, ↓ inflammation, ↓ oxidative stress, ↓ organ injury, ↓ apoptosis (rat) (84-88) Na_2S ↑ survival, ↓ resuscitative requirements, ↓ organ injury, ↓ inflammation (pig) (89, 90)	• Variable effects in large animals • Pulmonary and mitochondrial toxicity • Pro- and anti-inflammatory effects • H_2S easily lost from solution, slow-releasers not tested in shock • Efficacy differs with treatment onset Not effective in all studies (pig) (83)
Ghrelin	Decreases body temperature	↑ survival, ↑ cognitive function, ↓ inflammation (rat) (98)	• No effects in non-fasting rodents or healthy humans Not tested in larger animals
Adenosine/Lidocaine/ Magnesium (ALM)	Antiadrenergic, immunosuppressive, antioxidant, lowers cellular calcium entry, antiarrhythmic effects, improves coagulation	• ↑ survival, ↑ MAP, ↓ arrhythmias, ↑ glycocalyx integrity, ↓ clotting times (rat) (101-103) ↑ MAP, ↓ resuscitation requirements, ↓ lactic acidosis, ↑cardiac/ kidney function, ↑ urine output (pig) (104, 105)	Effect on survival not tested in larger animals
D-β-hydroxybutyrate/	Energy source, antioxidant activity, HDAC	• BHB ↓ apoptosis (rat) (153) • BHB ↑ MAP, ↓ lactic acidosis, ↓ inflammation, ↓	Low doses may have adverse

Treatment	Proposed Mechanism	Beneficial Effects	Disadvantages
Melatonin (BHB/M)	inhibition, immune-modulation	apoptosis (pig) (154) • Melatonin ↑ MAP, ↓ HR, ↓ organ injury, ↓ inflammation, ↓ oxidative stress, ↓ apoptosis (rat) (226, 227, 229) • Melatonin ↑ survival, ↓ resuscitation requirements (mouse) (228, 230) • BHB/M ↑ survival, ↑ glycocalyx integrity (rat) (103, 231) BHB/M ↑ survival (pig) (232)	effects
Therapeutic Hypothermia	Decreases metabolic rate and oxygen consumption, activation of pro-survival pathways, anti-inflammatory	• ↑ survival, ↑ MAP, ↓ HR, ↓ resuscitation requirements, ↓ lactate, ↓ organ injury, ↓ intestinal permeability, ↓ inflammation, ↓ oxidative stress (rat) (112-115) • ↑ survival, ↓ lactate, ↓ organ injury (pig) (116, 117, 260) • EPR: survival ≤ 60 min, ↓ neurological impairment, ↓ kidney injury (rat) (119) • EPR: survival ≤180 min with mild or no neurological deficit (dog) (120, 121) EPR: survival ≤120min with mostly no neurological impairment or organ dysfunction after 4 weeks (pig) (261)	• Experimental protocols may not be feasible in clinic • Optimal depth may vary with shock severity • Arrhythmias • Serum electrolyte changes • Insulin resistance • Reversible hypocoagulopathy • May increase risk for infection Slowed drug metabolism

Objectives

A combination of 4 M BHB/43 mM melatonin improves survival in animal model of hemorrhagic shock (231, 232). Despite these promising results, various steps remain on the path to clinical use of this treatment. The objective of the work presented here is to complete some of these steps. We conducted multiple experiments in *in vivo* and *in vitro* models of hemorrhagic shock and ischemia/reperfusion injury to evaluate the safety, optimize the dosing, administration and formulation, and to unravel the mechanism of action behind BHB/M.

Intravenous infusion is the most common route for the administration of resuscitation fluids. However, in the context of hemorrhagic shock, when blood volume is low, gaining intravenous access quickly can be difficult. This is especially true in complex, challenging situations like the battlefield. Consequently, the Institute of Medicine recommended the use of intraosseous infusion for the administration of resuscitation fluids in the battle field (33). Our first objective was to **evaluate the safety of intravenous and intraosseous 4 M BHB/43 mM M infusion in a porcine model of hemorrhagic shock and polytrauma** (Chapter 2). We hypothesized that BHB/M administered via either route of administration is safe.

Dosing optimization and evaluation of drug toxicity are essential steps in preclinical drug development. Our second objective was to **define the maximum tolerated dose of 4 M BHB/43 mM M in porcine hemorrhagic shock and polytrauma** (Chapter 3 Objective 1). We hypothesized that doubling the dose of BHB/M would not be associated with unacceptable adverse effects, while administration of four times the BHB/M standard dose would exert increased toxicity.

Due to the low aqueous solubility of melatonin, the 4 M BHB/43 mM M treatment solution contains 20% (v/v) DMSO. The parenteral use of DMSO has been controversial (262), and DMSO has been associated with different adverse effects, including emesis, anemia and intravascular hemolysis (246, 247, 263, 264). Recently published experiments utilizing a rat hemorrhagic shock model suggest that the concentration of melatonin (and therefore DMSO), but not BHB in the treatment could be decreased without loss of efficacy (233). The objective of our third experiment was to **test the efficacy of decreased melatonin concentrations in combination with 4 M BHB in our**

established porcine hemorrhagic shock, trauma and resuscitation model (Chapter 3 Objective 2). We hypothesized that solutions containing 4 M BHB and a melatonin concentration of 43 mM would be equally as effective at improving post-hemorrhagic shock survival as solutions with 4 M BHB in combination with 20 mM, 10 mM, 4.3 mM, or 0.43 mM melatonin.

Another important step in the preclinical development of BHB/M is the optimization of the treatment formulation. As described above, DMSO infusion has been associated with adverse effects. Furthermore, the current formulation requires a three-step reconstitution procedure, which constitutes a challenge for drug preparation in the field. The objective of our next experiment was to **generate and evaluate two novel BHB/M formulations void of DMSO** (Chapter 4). We hypothesized that these new solutions would be equally as effective as the original formulation in a rat hemorrhagic shock model. Indeed, non-DMSO solutions of BHB/M were equally effective as BHB/M/DMSO, with lower induction of intravascular hemolysis.

Lastly, although BHB/M has achieved promising results in preclinical hemorrhagic shock models, the mechanism of action behind the treatment has remained elusive. Therefore, the objective of our last set of experiments was to **unravel the mechanism of action behind BHB/M in an *in vitro* ischemia/reperfusion model** (Chapter 5). We hypothesized that BHB/M exerts beneficial effects by maintaining mitochondrial function throughout ischemia and decreasing oxidative stress upon reperfusion.

Chapter 2. Safety of D-β-Hydroxybutyrate and Melatonin for the Treatment of Hemorrhagic Shock with Polytrauma.

This chapter was published in:

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Synopsis

Introduction. D-β-hydroxybutyrate (BHB) and melatonin (M) treatment improves survival in animal models of hemorrhagic shock. Here, we evaluated the safety of BHB/M via two routes of administration in a porcine hemorrhagic shock/polytrauma model. Furthermore, we assessed BHB/M serum concentrations after intravenous and intraosseous infusion of different BHB/M doses in healthy pigs.

Methods. Pigs underwent pulmonary contusion, liver injury, and hemorrhage. Injured animals were treated with an intravenous or intraosseous bolus of BHB/M or lactated Ringer's solution (LR), followed by 4 h continuous infusion of the respective fluid (n=12 per group). Pigs were resuscitated with LR (1 h) and then LR and shed blood (20 h). Physiological data and blood samples were analyzed throughout the experiment. In a second study, we infused healthy pigs intravenously or intraosseously with BHB/M at three different doses (n=4 per group).

Results. There were no differences between groups in physiologic measurements (heart rate, mean arterial pressure, cardiac output), organ function markers (ALT, AST, BUN, CK, LDH), or histopathology. BHB/M treated animals exhibited transient changes in blood Na^+, K^+, pH and lactate. Differences in survival were not statistically significant. There was a trend towards decreased survival after intraosseous infusion, potentially related to lower circulating BHB and melatonin levels. Healthy pigs had higher drug serum concentrations after intravenous than after intraosseous infusion of BHB/M at the standard, but not the double dose.

Conclusion. BHB/M in doses previously shown to be associated with improved survival is safe in a porcine hemorrhagic shock/polytrauma model. Intravenous infusion is the preferred route of administration at standard doses.

Introduction

Hemorrhage control and fluid resuscitation are two fundamental aspects of hemorrhagic shock treatment. Although fluid replacement therapy has been used for decades, optimal resuscitation strategies remain a topic of ongoing debate. It has been increasingly recognized that the use of existing resuscitation fluids can lead to adverse effects (27). Partially due to these findings, the Institute of Medicine recommended the development and evaluation of new resuscitation fluids for acute treatment of massive blood loss (33). These recommendations include the modification of existing resuscitation solutions with ketone bodies, free radical scavengers and antioxidants.

During torpor, hibernating mammals experience metabolic changes similar to those in hemorrhagic shock, including decreased blood flow, oxygen consumption and metabolism (43). Interestingly, unlike patients suffering from severe blood loss, these animals can quickly return to a normal state of activity without signs of tissue damage or organ injury (265). Consequently, utilizing the mechanisms that prevent tissue damage during torpor and arousal from hibernation may help alleviate the detrimental effects of hemorrhagic shock.

In 2010, Klein and coworkers applied a strategy that exploited changes observed during hibernation and arousal in a rat model of hemorrhagic shock (231). Hibernating mammals exhibit elevated serum levels of the ketone body D-β-hydroxybutyrate (BHB) during torpor (47). Furthermore, serum concentrations of the antioxidant and free radical scavenger melatonin (M) spike during arousal (222, 266). In this study, treatment with a combination of BHB and melatonin resulted in significantly increased survival in rats subjected to severe blood loss followed by partial blood return (231). These findings were confirmed in a porcine model of hemorrhagic shock (232). Here, pigs were exposed to 60 minutes of severe blood loss, followed by standard resuscitation. After 15 minutes of shock, animals received a bolus of D-β-hydroxybutyrate/ melatonin (BHB/M) or control solution, followed by a 4-hour continuous infusion of the respective fluid at a decreased rate (232). Treatment with BHB/M resulted in significantly improved survival.

In the current publication, we used a porcine model of hemorrhagic shock with polytrauma to assess the safety of BHB/M treatment via two routes of administration: intravenous (IV) and intraosseous (IO) infusion. As intravenous access is often hindered

in patients with low blood volume, intraosseous infusion is the recommended alternative way to administer fluids (33).

We hypothesized that intravenous and intraosseous treatment with BHB/M would be safe. Interestingly, although both treatments did not cause severe adverse effects, infusion via the intraosseous route resulted in a trend towards decreased survival in this group. We therefore conducted a second experiment to test the hypothesis that the lower effectiveness of intraosseous BHB/M was due to decreased serum drug concentrations. Here, we evaluated serum levels of BHB and melatonin in healthy pigs treated with three doses of BHB/M via the intravenous and the intraosseous route.

Materials and methods

Animal preparation

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (IACUC). The care and handling of animals were in accord with the National Institutes of Health guidelines for ethical animal research.

Safety evaluation of intravenous and intraosseous administration

Instrumentation

Thirty-six (18 male, 18 female) Yorkshire-Landrace pigs (15-25 kg, Manthei Hog Farm, LLC, Elk River, MN) were fasted overnight prior to surgery but were allowed water *ad libitum*. We selected juvenile pigs to allow for administration of a low dose of BHB, which constituted a significant expense in the study. The pigs were anesthetized with telazol (4-6 mg/kg intramuscular) (Fort Dodge Animal Health, Fort Dodge, IA) and given ceftiofur (5 mg/kg IV). Anesthesia was maintained via intravenous propofol infusion (AstraZeneca Pharmaceuticals, Wilmington, VA), along with 80% inhaled nitrous oxide (232, 267). Pigs were intubated and ventilated (Servo 900C, Siemens-Elema, Sweden). FiO_2, inspiratory tidal volume and/or respiratory rate were adjusted to maintain a partial pressure of arterial oxygen (PaO_2) of 70 to 120 torr and a partial pressure of arterial carbon dioxide ($PaCO_2$) of 35 to 45 torr throughout anesthesia. Buprenorphine (0.03mg/kg subcutaneous.) was administered every 4 hours during anesthesia.

After exposure of the right femoral artery, a catheter was placed to allow for sample drawing and continuous blood pressure management. The right external jugular vein was surgically exposed and a 7 French introducer (Avanti, Cordis Corp., Miami Lakes, FL) and a Swan-Ganz catheter (5 French, Edwards Lifesciences, Irvine, CA) were placed for intermittent sampling of pulmonary artery occlusion pressure (pulmonary wedge pressure), thermodilution cardiac output (CO) and mixed venous blood. A midline laparotomy was conducted. A splenectomy, which is commonly performed in porcine models of hemorrhagic shock, was conducted to prevent variable autotransfusion of blood not amenable to quantification in our study (268).

We placed catheters into the urinary bladder (via a stab cystotomy) and into the inferior vena cava (IVC). After surgical preparation, the pig was allowed to stabilize until

blood lactate levels were 2.0 mg/dl or less, after which baseline (B) samples were collected.

Hemorrhagic shock with polytrauma model

An overview over the experimental protocol is depicted in Figure 2.1. Shock with polytrauma was initiated by the induction of a blunt chest injury with a captive bolt gun (right side). The typical injury pattern observed consisted of 2-5 rib fractures with underlying pulmonary contusion without significant hemothorax. After lung contusion, hemorrhagic shock was induced by withdrawal of blood from the IVC until a systolic blood pressure of below 60 mm Hg was reached (35%–50% of total blood volume withdrawn). Shed blood was stored in an acid-citrate-dextrose bag for subsequent use during resuscitation. Two liver crush injuries were then created in the peripheral liver parenchyma using a Holcomb clamp technique (269). 15 minutes after pulmonary contusion, to simulate arrival of field health care personnel, animals received a bolus (1 ml/kg over 10 min) of either BHB/M or lactated Ringer's solution (Baxter, Deerfield, IL), followed by a continuous 4-hour infusion of the respective fluid (0.66 ml/kg/hour). BHB/M solution contained 4 M BHB (Lonza, Basel, Switzerland), 43 mM melatonin (Flamma S.p.A., Chignolo, Italy), and 20% DMSO (Alfa Aesar, Ward Hill, MA). Lactated Ringer's solution (LR) was infused via intravenous catheter (n=12), while BHB/M was administered either intravenously (n=12) or by intraosseous infusion into the left proximal tibia (n=12). Intraosseous access was obtained using an EZ-IO® intraosseous infusion system.

After 45 minutes of shock (S45 min), a 60-minute limited resuscitation (R) protocol was utilized to simulate transport time to standard of care facility. Animals were evaluated every ten minutes and received boluses of LR (20 ml/kg IV) to maintain a systolic blood pressure of >80 mmHg. After 60 minutes of limited resuscitation, a full resuscitation (FR) protocol was utilized to simulate common hospital resuscitation practice (Figure 2.1 B). Briefly, animals were evaluated every ten minutes and, if necessary, resuscitated to standard clinical endpoints of systolic blood pressure (≥90 mm Hg), urine output (≥1 ml/kg/h), and hemoglobin (≥6 g/dl) via infusion of intravenous boluses of LR (20 ml/kg) or shed blood (10 ml/kg). Glucose levels were maintained with intravenous bolus infusion of 25 mL of 50% dextrose for glucose levels < 60 mg/dl. At 22 hours from initiation of injury, the animals were extubated and returned to post-op for

recovery. All pigs were monitored post operatively in accordance with IACUC regulations. Animal care staff performed checks on wellness, body temperature and respiration, and administered pain medication (ketoprofen (2 mg/kg daily), buprenorphine (0.03 mg/kg twice daily)).

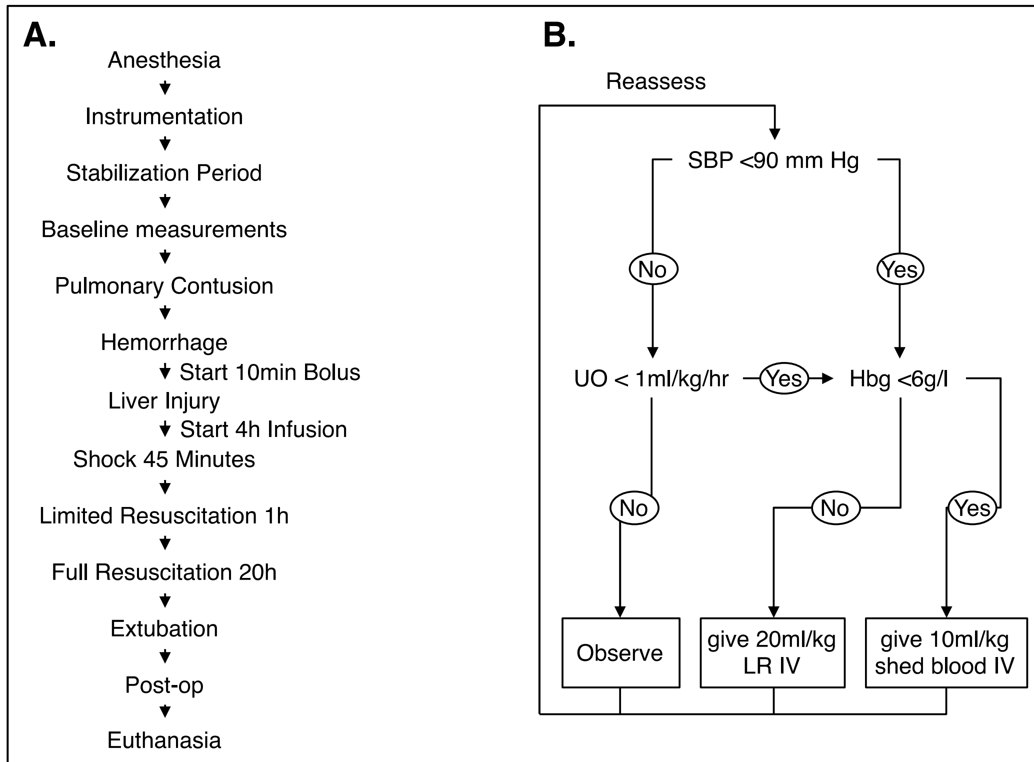

Figure 2.1. (A) Shock, injury and resuscitation protocol and (B) full resuscitation protocol.

Hemodynamic and physiologic measurements

Physiologic parameters were monitored continuously throughout the first 22 hours of the experiment (including heart rate, arterial pressure, pulmonary artery pressure, body temperature and BIS score). Additional invasive hemodynamic parameters were measured at baseline, during shock, at the end of the shock period and then

approximately every hour for 20 hours after the initiation of resuscitation. These included CO, pulmonary wedge pressure, bladder pressure, and urine output.

Arterial and venous blood were analyzed via blood gas analyzer (Instrumentation Laboratory Co., Lexington, ME) for measurement of PaO2, PaCO2, mixed venous oxygen saturation (SvO2), partial pressure of venous carbon dioxide (PvCO2), hemoglobin (Hgb), base excess, blood pH, blood electrolyte, glucose and lactate levels.

Organ function was assessed at different time points throughout the course of the experiment by measuring blood bilirubin (via bichromatic endpoint), albumin (via polychromatic endpoint), total protein, alkaline phosphatase alanine aminotransferase, aspartate aminotransferase, urea nitrogen, creatinine kinase and lactate dehydrogenase (all via bichromatic rate) levels. Blood samples were analyzed in the Clinical Laboratory Improvement Amendments (CLIA)-certified Fairview Diagnostics Laboratory. A modified veterinary Glasgow Coma Score (GCS) was obtained at four time points throughout the course of the experiment for assessment of neurologic status. Surviving animals were sacrificed three or 14 days after the induction of shock.

After death, a pathologist examined all animals to evaluate organ injury due to shock, trauma or treatment. Histopathology tissue slices were stained with hematoxylin and eosin. Sites of gross and histopathological examination include adrenal glands, bladder, bones, brain, brain stem, cerebellum, esophagus, eyes, gallbladder, heart and great vessels, small and larger intestine, left and right kidney, left and right lateral liver lobes, mesenteric and submandibular lymph nodes, left and right lung, oral cavity and tongue, pancreas, pituitary gland, reproductive tract, ribs, skin, spinal cord, sternum bone marrow, stomach, thymus, thyroid, trachea and bronchi, and urinary bladder.

Pharmacokinetic dosing study

A total of twenty-eight (14 male, 14 female) Yorkshire-Landrace pigs (15-25 kg, Manthei Hog Farm, LLC, Elk River, MN) were fasted overnight before surgery but were allowed water *ad libitum*. The pigs underwent the same instrumentation procedure, pain management, stabilization period and baseline measurements as in the previous experiment without induction of injury or hemorrhagic shock and without resuscitation. 15-30 minutes after baseline measurements animals received a bolus (infused over 10 minutes) of either BHB/M (4 M D-β-hydroxybutyrate, 43 mM melatonin and 20%

DMSO) or LR via a central intravenous or tibial intraosseous catheter, followed by 4 h continuous BHB/M or LR infusion at the appropriate rate (Table 2.1, n=4 per group). All animals were sacrificed eight hours after the start of BHB/M or LR infusion.

Similar to the previous experiment, invasive hemodynamic parameters and markers of organ function were assessed at baseline, 30 minutes after the start of bolus infusion and then every hour (invasive hemodynamics) or at key time points (organ markers) until euthanasia. The animals receiving the double dose of BHB/M intravenously or intraosseously, as well as pigs treated with LR, were necropsied and histopathological changes were assessed as described above. One pig (BHB/M 2x IO) was excluded from the analysis due to complications with the infusion.

Table 2.1. Treatment groups in the pharmacokinetic dosing study.

Treatment	Volume	Dose
Lactated Ringer's	1 ml/kg bolus 0.66 ml/kg/h infusion	n.a. n.a.
BHB/M 0.5x IV	0.5 ml/kg bolus 0.33 ml/kg/h infusion	206 mg BHB 5 mg melatonin/kg 136 mg BHB 3.3 mg melatonin/kg/h
BHB/M 1x IV	1 ml/kg bolus 0.66 ml/kg/h infusion	412 mg BHB 10 mg melatonin/kg 272 mg BHB 6.6 mg melatonin/kg/h
BHB/M 2x IV	2 ml/kg bolus 1.32 ml/kg/h infusion	824 mg BHB 20 mg melatonin/kg 544 mg BHB 13.2 mg melatonin/kg/h
BHB/M 0.5x IO	0.5 ml/kg bolus 0.33 ml/kg/h infusion	206 mg BHB 5mg melatonin/kg 136 mg BHB 3.3 mg melatonin/kg/h
BHB/M 1x IO	1 ml/kg bolus 0.66 ml/kg/h infusion	412 mg BHB 10 mg melatonin/kg 272 mg BHB 6.6 mg melatonin/kg/h
BHB/M 2x IO	2 ml/kg bolus 1.32 ml/kg/h infusion	824 mg BHB 20 mg melatonin/kg 544 mg BHB 13.2 mg melatonin/kg/h

BHB and melatonin analysis

Sample extraction

D-ß-hydroxybutyrate and melatonin serum concentrations were analyzed at various time points throughout both experiments. Porcine serum samples (0.02 mL) were extracted by protein precipitation with addition of 0.2 mL of ice-cold acetonitrile fortified with internal standard (5 ng of melatonin–d4 (Toronto Research Chemicals, Toronto, Ontario, CAN) and 5,000 ng of gHB-d6 (Cerilliant (Round Rock, TX)). The samples were centrifuged at 10,000 g (5 min, 4 °C). Aliquots of the supernatant were evaporated to dryness using a nitrogen evaporator (Zymark Turbo Vap LV, Hopkinton, MA) set at 37°C. For melatonin analysis, samples were reconstituted with 100 µl of mobile phase (50:50, 0.1% formic acid in deionized water: 0.1% formic acid in methanol). BHB samples tubes were derivatized with 100 µl of BSTFA:pyridine (5:1) (30 min, 70 °C).

Analysis

Quantification of melatonin was performed using an Acquity UPLC ultrahigh-performance liquid chromatograph (Waters, Milford MA) coupled with a Quattro Ultima triple stage quadrupole mass spectrometer (Micromass, Manchester, United Kingdom). Chromatographic separation was performed with a BEH C18, reversed phase UPLC column (50x2.1 mm, particle size 1.7 µm, Waters, Milford, MA). Quantification of BHB was performed using Hewlett-Packard 5971 MSD coupled with a 5890 gas chromatograph (GC-MS). The chromatographic separation was performed with an Rx-5 ms capillary column (30 m x 0.25 mm, Restek, Bellefonte, PA). Quantification was performed with peak area ratios obtained from multiple reaction monitoring (melatonin) or selected ion monitoring (BHB) and were utilized for the construction of calibration curves, using weighted (1/x) linear least-squares regression of the serum concentrations and the measured peak area ratios. Data collection, peak integration, and calculations were performed using QuanLynx (melatonin) or HP Chemstation and Chromperfect (BHB) software. Assay accuracy and variability were 98.6% and 4.4% (melatonin) or 106.1% and 2.9% (BHB), respectively.

Data analysis and statistical methods

Kaplan-Meier analysis with Generalized Wilcoxon test was used to analyze survival. Area under the curve was calculated with PKSolver, from the start of drug administration

to the last sampling time point ($AUC_{0\text{-FR 20h}}$) using the trapezoidal rule (270). After assessing normality, we used Mann Whitney U test with Bonferroni corrections, or Kruskal Wallis test with Dunn-Bonferroni corrections for the comparison of single time points. Data are reported as medians with interquartile ranges (IQR), an adjusted p value <0.05 was considered significant. We used SPSS version 22.0 for Macintosh (SPSS, Chicago, IL) for all statistical analyses.

Results

Safety evaluation of intravenous and intraosseous administration

Shock induction and resuscitation

There were no significant group differences in animal weights (median weight 19.3 kg [18.3-20.8] LR, 19.7 kg [18.7-20.1] BHB/M IV, 19.5 kg [17.9-20.0] BHB/M IO). All animals were injured and resuscitated similarly as there was no significant difference in the amount of blood removed during shock induction (24.5 ml/kg [24.5-27.7] LR, 24.5 ml/kg [24.5-24.6] BHB/M IV, 24.5 ml/kg [24.1-24.6] BHB/M IO), the total amount of blood received during resuscitation (574 ml [548-590] LR, 571 ml [483-603] BHB/M IV, 536 ml [0-573] BHB/M IO) or the amount of fluids administered at any time point throughout the experiment (data not shown). The net fluid intake/output was not significantly different between groups at most time points throughout the experiment (Figure 2.2).

Physiology

We did not observe significant differences in heart rate, mean arterial pressure, cardiac output, systemic oxygen consumption, and mixed venous oxygen saturation between treatment groups throughout the experiment (Table 2.2). Animals treated with LR had significantly higher hemoglobin levels at the end of the shock period. In accordance with our previous study (232), lactate levels were significantly increased in pigs receiving BHB/M when compared to LR-treated animals from FR 3h-FR 5h (Table 2.2 and Figure 2.3).

Levels of various markers of organ function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, alkaline phosphatase, albumin, blood urea nitrogen (BUN), total creatine kinase (CK), lactate dehydrogenase (LDH)) were not significantly different between treatment groups at key time points during injury, resuscitation and follow up until euthanasia (Table 2.3). We saw significantly higher total serum protein concentrations in the control group at S 45min (versus BHB/M IV) and at FR 20h (versus BHB/ IO). Porcine Glasgow coma scores were not significantly different between groups during follow-up (data not shown).

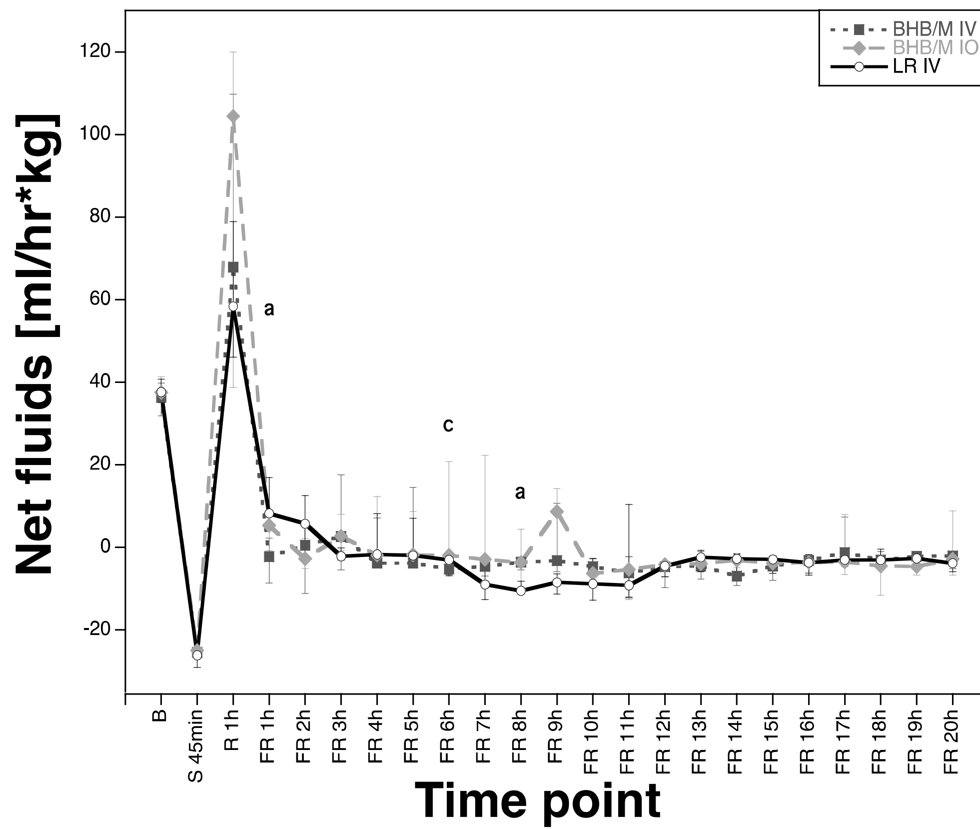

Figure 2.2. Net fluids per weight in pigs exposed to hemorrhagic shock and injury. Net fluids were calculated as the amount of fluid administrated hourly (LR or blood) during surgery or resuscitation minus by the amount of fluids lost (blood or urine) over the last hour divided by bodyweight. Data are presented as medians (IQR). n = 12 for all groups at the beginning of the experiment. a $p < 0.05$ BHB/M IV – LR IV; c $p < 0.05$ BHB/M IV – BHB/M IO.

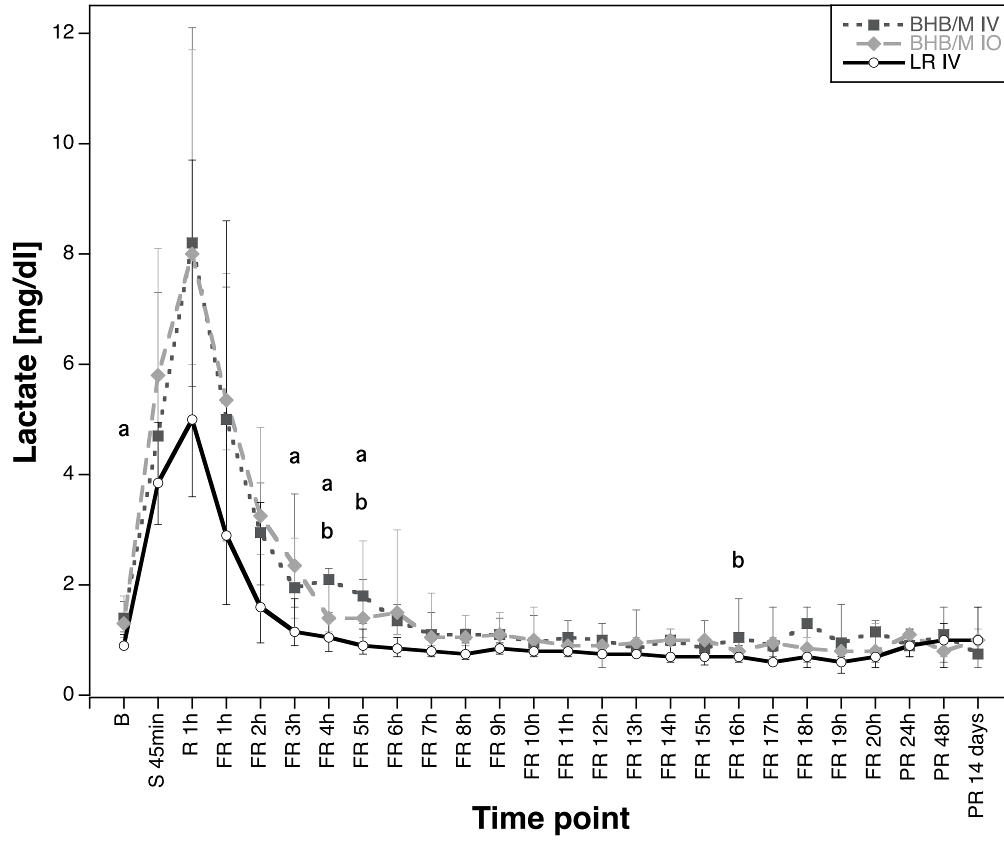

Figure 2.3. Lactate blood concentrations in pigs exposed to hemorrhagic shock and injury. Error bars depict IQR. N = 12 for all groups at the beginning of the experiment. a p<0.05 BHB/M IV – LR IV; b p<0.05 BHB/M IO – LR IV.

Table 2.2: Physiologic and hemodynamic measurements in pigs exposed to hemorrhagic shock and injury.

	B			S 45 min			FR 2 h			FR 20 h			PR 14 d		
	LR	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**
Heart rate (beats/min)	113 (93-135)	126 (112-136)	126 (113-148)	249 (236-254)	203 (157-254)	241 (174-248)	166 (133-199)	130 (117-189)	162 (131-234)	157 (127-170)	137 (107-164)	155 (132-163)	126 (124-126)	133 (116-153)	112 (83-112)
MAP (mm Hg)	87.9 (82.5-95.0)	84.7 (70.9-91.2)	87.5 (79.2-97.6)	42.4 (36.6-51.2)	42.3 (32.3-44.0)	38.3 (32.0-46.0)	77.3 (68.6-75.2)	71.3 (66.0-82.2)	77.2 (69.1-85.0)	76.0 (68.6-84.1)	68.5 (64.3-72.7)	70.7 (54.3-87.3)	71.0 (62.3-71.0)	86.2 (78.0-93.8)	102.3 (86.3-102.3)
CO (l/min)	2.8 (2.5-3.1)	2.9 (2.5-3.5)	2.6 (2.1-3.5)	1.7 (1.2-1.8)	1.6 (1.0-2.2)	1.1 (0.7-1.6)	4.6 (3.5-5.9)	4.8 (4.5-6.0)	4.5 (3.4-4.8)	4.4 (3.7-4.8)	3.9 (2.9-5.5)	3.8 (3.3-5.7)	n.d.	n.d.	n.d.
VO_2 (ml O_2/min)	4.5 (3.8-4.7)	3.9 (3.3-5.5)	4.2 (3.4-4.6)	4.9 (4.0-5.6)	3.8 (3.4-4.7)	4.3 (2.0-5.2)	4.8 (4.0-5.7)	5.2 (4.4-5.9)	4.8 (4.0-5.7)	4.5 (3.7-5.0)	4.6 (3.5-5.6)	5.5 (4.6-6.9)	n.d.	n.d.	n.d.
SvO_2 (ml O_2/min)	72.0 (70.0-75.3)	75.0 (60.8-79.5)	73.5 (65.0-77.3)	28.5 (19.3-34.8)	29.0 (14.0-44.0)	21.0 (14.0-31.0)	72.5 (62.3-77.5)	72.0 (68.8-75.5)	68.9 (63.0-74.3)	69.0 (56.8-75.3)	68.0 (59.5-77.3)	71.5 (55.0-73.8)	76.0 (59.0-76.0)	80.5 (72.8-87.0)	55.0 (52.0-55.0)
Base Excess	5.2 (0.3-7.1)	5.7 (3.4-7.4)	4.7 (-0.2-5.3)	0.4 (-4.8-2.2)	2.6 (-0.5-6.8)	-0.4 (-4-2.5)	3.3 (-5.9-7.9)	13.9* (5.8-17.3)	7.0 (-0.5-12.6)	4.0 (1.5-6.1)	5.5 (3.6-7.7)	6.0 (-1.0-6.8)	-9.7 (-12- -9.7)	9.0 (4.5-11.5)	9.4 (3.3-9.4)
Hgb (g/dl)	8.7 (8.2-9.0)	8.7 (7.9-9.2)	8.1 (7.8-8.4)	7.1 (6.8-7.3)	6.2* (5.6-6.5)	6.2* (6.2-6.8)	6.2 (5.7-6.4)	6.2 (5.6-6.7)	5.9 (5.5-5.9)	5.6 (4.9-6.2)	5.9 (5.1-6.4)	5.8 (5.4-6.5)	8.7 (8.1-8.7)	8.7 (8.6-9.3)	9.3 (8.1-9.3)

	B			S 45 min			FR 2 h			FR 20 h			PR 14 d		
	LR	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**
Body temperature (°C)	37.8 (37.5-38.8)	37.7 (37.3-38.4)	38.1 (37.5-38.4)	38.3 (37.9-39.2)	38.0 (37.6-38.9)	38.4 (37.9-38.9)	38.8 (38.5-39.2)	38.3 (37.7-38.9)	38.6 (38.1-39.5)	39.5 (39.1-39.9)	38.9^{+} (38.6-39.4)	39.7 (39.4-39.9)	n.d.	n.d.	n.d.
Lactate (mg/dl)	0.9 (0.8-1.1)	1.4* (1.1-1.8)	1.3 (1.0-1.9)	3.9 (3.1-5.0)	4.7 (3.9-7.3)	5.8 (4.7-8.1)	1.6 (0.9-4.3)	3.0 (1.9-4.4)	3.3 (2.5-5.2)	0.7 (0.5-0.8)	1.2 (0.6-1.3)	0.8 (0.7-1.3)	1.0 (0.8-1.0)	0.8 (0.5-1.0)	1.0 (1.0-1.0)

Data is presented as median (IQR). n = 12 for all groups at the beginning of the experiment. * $p<0.05$ versus LR at the same time point; $^{+}$ $p<0.05$ versus BHB/M IO at the same time point.

Table 2.3. Markers of organ damage in pigs exposed to hemorrhagic shock and injury.

	B			S 45 min			FR 2 h			FR 20 h			PR 14 d		
	LR	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**
AST (U/dl)	26.0 (20.5-37.0)	27.0 (25.3-41.8)	31.5 (20.3-41.8)	68.0 (51.0-128.0)	63.0 (54.0-93.0)	90.0 (72.8-159.5)	127.5 (96.3-183.8)	188.5 (159.8-209.5)	184.0 (148.0-253.0)	73.5 (55.3-90.8)	111.0 (85.0-163.5)	107.0 (69.0-130.0)	27.0 (26.0-27.0)	32.5 (27.8-38.3)	36.0 (28.0-36.0)
ALT (U/dl)	61.0 (56.5-78.0)	71.0 (63.0-91.5)	72.5 (60.0-85.5)	64.0 (59.0-76.0)	67.0 (60.0-74.0)	74.5 (60.0-84.5)	75.0 (64.3-81.5)	75.0 (68.0-87.3)	77.0 (68.0-82.0)	78.5 (66.3-82.3)	80.0 (67.8-91.5)	75.0 (68.0-86.0)	67.0 (57.0-67.0)	67.0 (61.5-81.8)	91.0 (69.0-91.0)
Bilirubin (mg/dl)	0.10 (0.10-0.10)	0.10 (0.10-0.10)	0.10 (0.10-0.175)	0.10 (0.10-0.10)	0.10 (0.10-0.10)	0.10 (0.10-0.125)	0.10 (0.10-0.10)	0.10 (0.10-0.10)	0.10 (0.10-0.20)	0.10 (0.10-0.23)	0.10 (0.10-0.10)	0.10 (0.10-0.30)	0.10 (0.10-0.10)	0.10 (0.10-0.10)	0.10 (0.10-0.10)
Alk Phos (U/dl)	218.0 (187.8-293.8)	231.5 (173.8-269.5)	221.5 (182.0-249.5)	206.0 (166.0-262.0)	179.0 (158.0-212.0)	197.5 (134.3-219.8)	178.5 (135.5-255.0)	183.5 (154.0-212.8)	187.0 (162.0-200.0)	241.0 (154.8-265.3)	194.5 (167.3-274.3)	192.0 (152.0-249.0)	157.0 (117.0-168.0)	140.5 (126.3-251.5)	176.0 (87.0-176.0)
Albumin (g/dl)	2.2 (2.2-2.4)	2.4 (2.2-2.5)	2.2 (2.1-2.2)	2.0 (1.9-2.1)	1.9 (1.8-2.0)	1.8 (1.7-2.0)	1.8 (1.7-2.0)	1.8 (1.7-2.0)	1.7 (1.5-1.9)	1.9 (1.7-1.9)	1.7 (1.5-1.7)	1.5 (1.4-1.8)	2.5 (2.2-2.5)	2.7 (2.5-2.8)	2.8 (2.7-2.8)
Total protein (g/dl)	4.6 (4.2-4.8)	4.6 (1.3-4.8)	4.5 (4.1-4.8)	4.0 (3.9-4.1)	3.5[*] (3.3-3.8)	3.9 (3.3-4.0)	3.5 (3.3-3.8)	3.4 (3.2-3.6)	3.4 (3.1-3.5)	3.8 (3.3-4.0)	3.5 (3.1-3.6)	3.1[*] (3.0-3.5)	5.1 (4.9-5.1)	5.2 (5.0-5.3)	5.0 (5.0-5.0)
BUN (mg/dl)	9.0 (7.3-9.0)	8.0 (7.3-11.3)	8.0 (7.0-9.8)	11.3 (10.0-12.0)	11.0 (9.0-13.0)	9.9 (9.8-11.3)	13.0 (11.0-14.0)	12.5 (10.8-14.0)	11.0 (10.0-15.0)	13.0 (11.8-15.0)	14.5 (11.8-16.0)	13.0 (12.0-16.0)	10.0 (8.0-10.0)	10.0 (8.0-12.0)	9.0 (4.0-9.0)

	B			S 45 min			FR 2 h			FR 20 h			PR 14 d		
	LR	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**	**LR**	**BHB/M IV**	**BHB/M IO**
CK (U/dl)	438.5 (384.5-536.8)	426.0 (345.0-581.0)	505.5 (410.8-565.0)	657.0 (571.0-773.0)	587.0 (458.0-692.0)	737.5 (621.5-891.8)	1355.0 (1259.5-2370.3)	1355.5 (1194.5-2867.0)	2484.0 (1209.0-3678.0)	1825.5 (1261.5-2815.5)	2858.5 (2196.0-4201.8)	1496.0 (1351.0-4209.0)	424 (201.0-424.0)	934.0 (199.8-4294.8)	680.0 (362.0-680.0)
LDH (U/dl)	1019 (964-1448)	1066 (957-1177)	1110 (1000-1269)	1231 (1050-1857)	1074 (968-1167)	1285 (1148-1680)	1815 (1472-2629)	2425 (1983-2657)	2594 (2108-2889)	2439 (1902-3259)	2572 (2285-3404)	3312 (2401-3701)	1672 (928-1672)	1332 (1109-1761)	1730 (1121-1730)

Data is presented as median (IQR). n = 12 for all groups at the beginning of the experiment. * $p<0.05$ versus LR at the same time point.

A pathologist further evaluated organ damage via gross organ and histopathological examinations. Typical findings were cross-shaped incisions of the right and left lateral liver lobes (sometimes associated with blood clots), dark purple lesions of the left and right lung (sometimes associated with broken ribs), incisions of the skin and some tracheal necrosis. These findings were in accordance to the surgery/injury/intubation protocol. We did not identify qualitative differences in the findings between treatment groups. Histopathological analysis detected collapsed alveolar spaces (commonly 25-50%), parenchymal disruption and locally extensive hepatocellular necrosis associated with hemorrhage in the liver (sometimes with some evidence of regeneration), tracheal mucosal necrosis, and sometimes mucosal edema. The pathologist ruled these findings as consistent with the experimental interventions. There were no qualitative differences in histopathological findings between treatment groups.

At the end of the shock period (30 min after start of bolus infusion), sodium levels were significantly increased in animals receiving BHB/M via either route (Figure 2.4 A). BHB/M treatment resulted in mild to moderate hypernatremia, with highest median sodium concentrations of 149 mEq/l at FR 2 h. While pigs receiving BHB/M IO also had significantly increased sodium serum levels, the effect was less prominent and shorter lasting than in animals treated intravenously.

Shock-induction was followed by an increase in blood potassium levels, which was less prominent in BHB/M IV animals than in the other groups (Figure 2.4 B). BHB/M IV pigs experienced moderate-to-severe hypokalemia with median potassium concentrations above 2.95 mEq/l at the start of the resuscitation phase. Median blood potassium levels remained significantly lower in pigs receiving intravenous BHB/M than in those receiving LR over the first seven hours of resuscitation. Again, this effect was less prominent in animals that were treated intraosseously, which is illustrated by the observation that for this group, the difference in K^+ levels was only significant at the end of the shock period.

Shock and injury resulted in mild-to-moderate acidosis in LR-treated animals (Figure 2.4 C). With ongoing resuscitation, pH increased until all three groups experienced moderate alkalosis. There was a trend towards increased blood pH in pigs receiving BHB/M IV when compared to the LR group, which reached significance after infusion completion (FR 3 h) and resolved during the resuscitation period (Figure 2.4 C). In

accordance with the observed changes in blood pH, BHB/M IV pigs exhibited significantly higher base excess at FR 1 h-FR 6 h (Table 2.2 and data not shown). Pigs treated with BHB/M intraosseously showed increases in pH and base excess that were less pronounced, rendering them statistically insignificant.

BHB/M IV pigs had significantly higher urine output than animals treated with LR during the first hours of resuscitation (Figure 2.5 A). Cumulative urine output was also higher in BHB/M IV pigs during the complete resuscitation period, although differences were only significant during the first third of the resuscitation phase (data not shown). There was no significant difference between pulmonary wedge pressures in BHB/M and LR treated pigs (Figure 2.5 B).

BHB/M serum concentrations

As expected, BHB/M-treated pigs had significantly higher BHB and melatonin serum concentrations than pigs treated with LR during the whole resuscitation phase (Figure 2.6). Interestingly, BHB/M pigs infused via the intravenous route showed higher BHB and melatonin serum levels than those receiving BHB/M intraosseously, although the differences were not statistically significant. The described trends are best observed when one examines the peak serum concentrations (C_{Max}) and the total drug exposure over time (area under the curve, AUC) for the respective groups (Figure 2.7). For both BHB and melatonin, these parameters were significantly higher in BHB/M IV and BHB/M IO animals than in those receiving LR. Furthermore, C_{Max} and AUC were higher in BHB/M IV than in BHB/M IO animals, although the differences were not statistically significant (Figure 2.7).

Survival

Survival between groups was not statistically significant (p=0.141). 48 hours after shock and injury induction, there was a trend towards increased survival in pigs receiving BHB/M intravenously, while animals treated with BHB/M via intraosseous infusion showed decreased survival (7/12) compared to LR IV (9/12) or BHB/M IV treated pigs (**Fig. 6**). All animals alive 48 hours after shock survived until the end of the experiment.

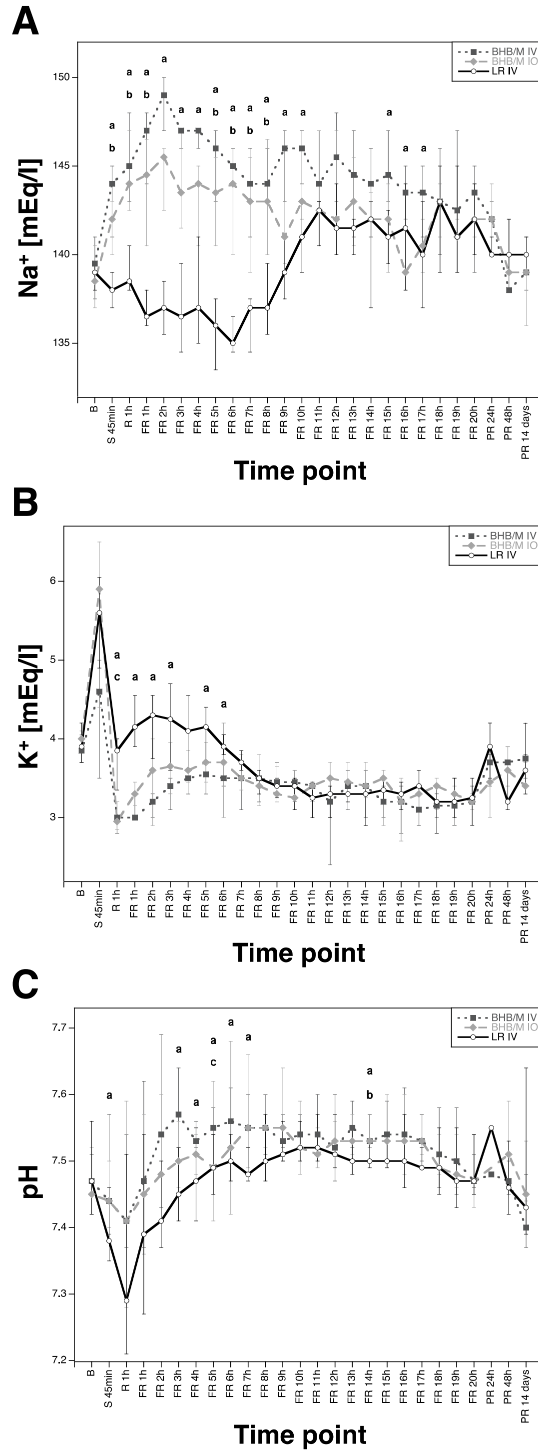

Figure 2.4. (A) Sodium and (B) potassium blood concentrations and (C) pH levels in pigs exposed to hemorrhagic shock and injury. Error bars depict IQR. n = 12 for all groups at the beginning of the experiment. a $p< 0.05$ BHB/M IV – LR IV; b $p< 0.05$ BHB/M IO – LR IV; c $p<0.05$ BHB/M IV – BHB/M IO.

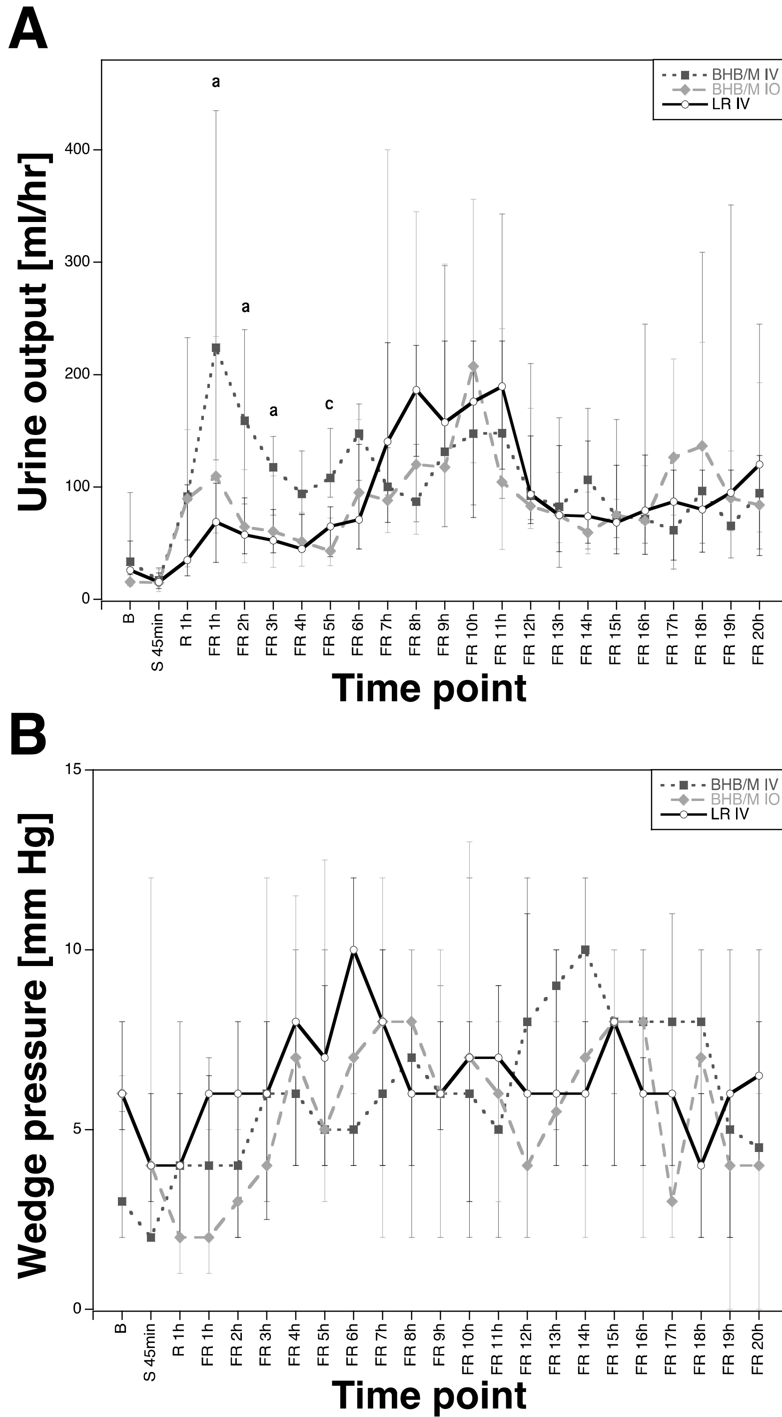

Figure 2.5. (A) Urine output and (B) pulmonary wedge pressure in pigs exposed to hemorrhagic shock and injury. Error bars depict IQR. n = 12 for all groups at the beginning of the experiment. a $p < 0.05$ BHB/M IV – LR IV; c $p < 0.05$ BHB/M IV – BHB/M IO.

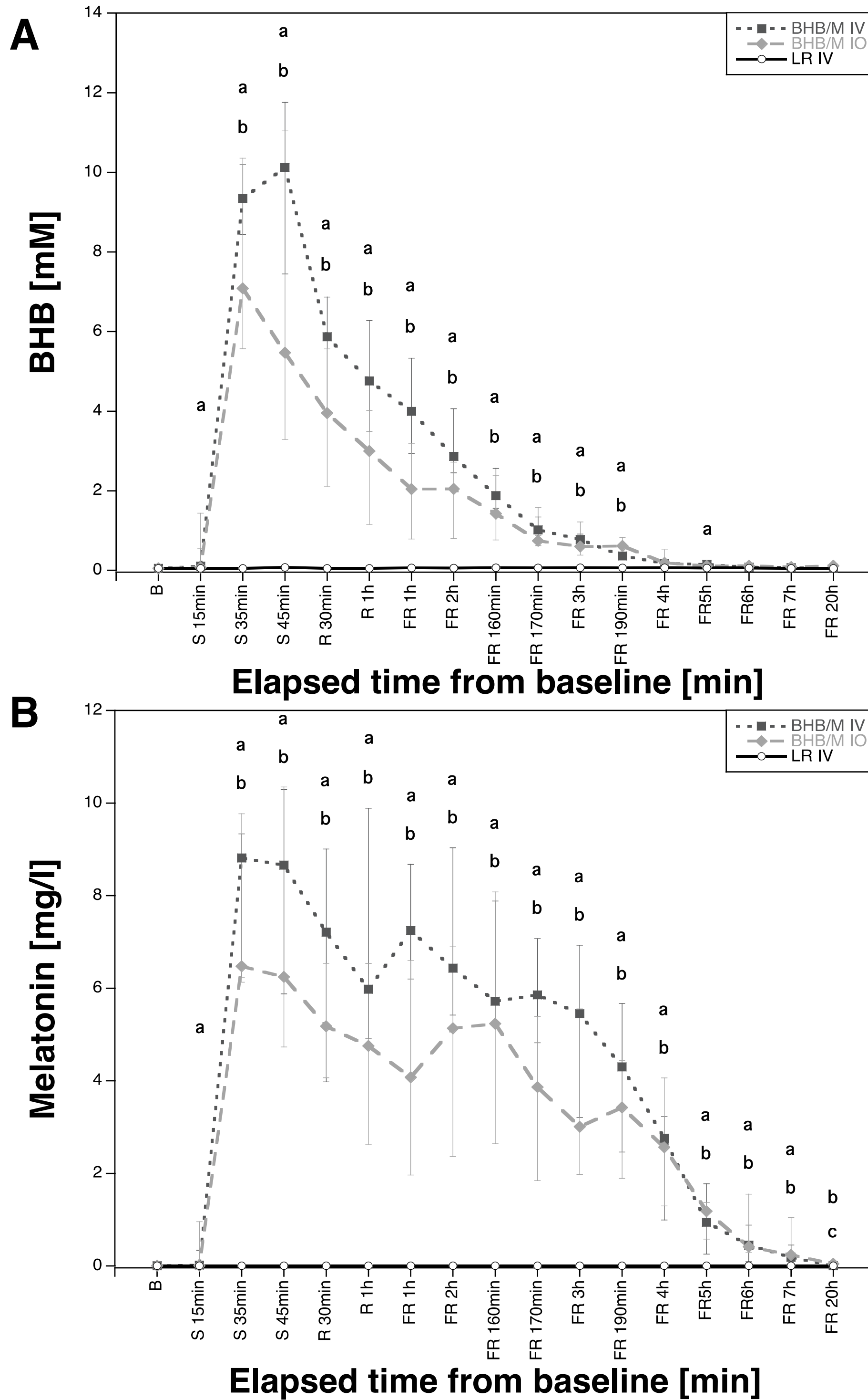

Figure 2.6. (A) BHB and (B) melatonin serum concentrations in pigs exposed to hemorrhagic shock an injury. Error bars depict IQR. N = 12 for all groups at the beginning of the experiment. a $p<0.05$ BHB/M IV – LR IV; b $p<0.05$ BHB/M IO – LR IV; c $p<0.05$ BHB/M IV – BHB/M IO.

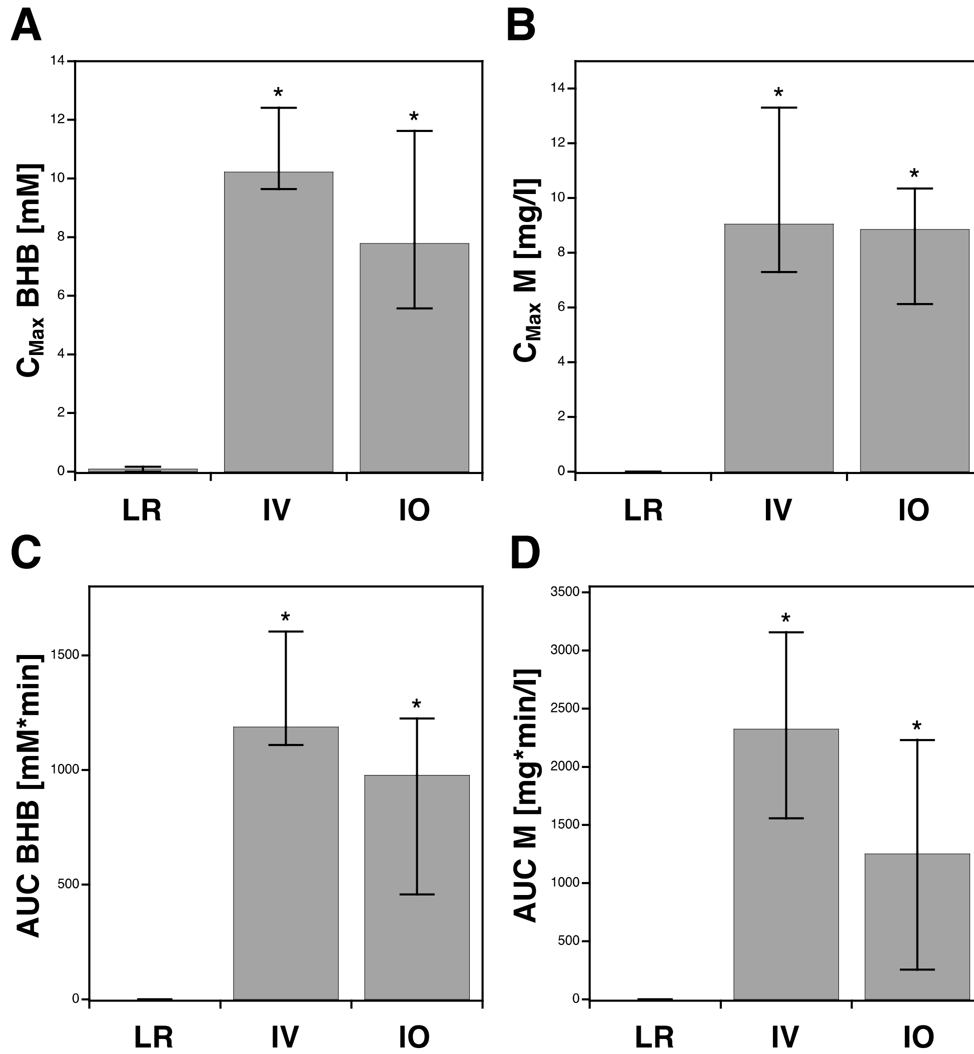

Figure 2.7. (A, B) Peak serum concentrations (C_{Max}) and (C, D) area under the curve (AUC) of D-ß-hydroxybutyrate and melatonin in pigs exposed to hemorrhagic shock and injury. Error bars depict IQR. C_{Max} was calculated from pigs alive after S 45 min (n=11 for BHB/M groups, n=12 for LR group). AUC was calculated from pigs alive at FR 20 h (n=11 for BHB/M IV, n= 7 for BHB/M IO, n=9 for LR). * $p< 0.05$ BHB/M – LR IV.

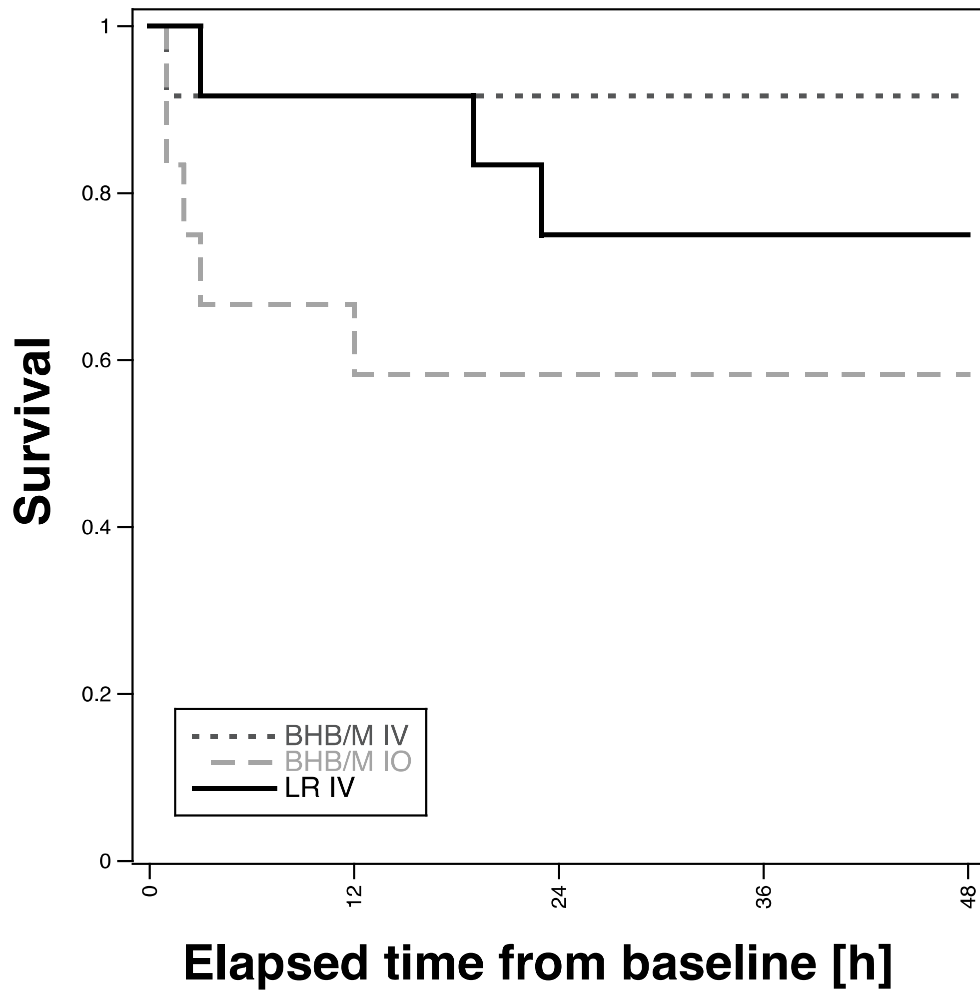

Figure 2.8. Kaplan-Meier Survival curve of in pigs exposed to hemorrhagic shock and injury.

Pharmacokinetic dosing study

Serum concentrations

All pigs treated with BHB/M showed rapid increases in drug serum concentrations (Figure 2.9). Intravenous infusion of half of the BHB/M standard dose resulted in significantly higher BHB peak serum concentrations (C_{Max}) than administration via the intraosseous route (Figure 2.10 A). There was no significant difference between melatonin C_{Max} and BHB and melatonin drug exposure over time (Figure 2.10 B-D). At the standard dose, pigs had significantly higher BHB C_{Max} and AUC levels after intravenous than after intraosseous infusion. We observed the same trend for melatonin; however, differences were not statistically significant. Interestingly, when pigs were infused with the highest dose of BHB/M (two times the standard dose), we did not observe a significant difference in peak serum levels or AUCs between groups (Figure 2.9). Rather, there was a trend towards higher BHB and melatonin C_{Max} and AUCs after intraosseous than after intravenous administration.

Physiology

The same physiologic variables and markers of organ function as in the first experiment were assessed at baseline and then hourly until euthanasia after FR 7 h. We did not observe significant differences between groups for these parameters throughout the experiment (data not shown). Median lactate concentrations remained at ≤2.1 mg/dl for all groups at all time points (data not shown). As in the previous study, a pathologist conducted a gross and histopathological examination upon euthanasia. All animals appeared to be in good health at euthanasia and no changes inconsistent with the experimental interventions were observed. There were no differences in qualitative findings between treatment groups.

Pigs treated with BHB/M showed dose dependent changes in blood pH and electrolyte levels (Figure 2.11). At the standard intravenous dose, increases in blood sodium levels were comparable to those in the initial safety study, resulting in mild-to-moderate hypernatremia (Figure 2.11 A). Sodium was significantly increased at most time points in animals receiving the double dose via either route of administration, resulting in moderate-to-severe hypernatremia with median sodium concentrations of 163 mEq/l or less.

Dose-dependent decreases in potassium blood concentrations resulted in mild-to-moderate hypokalemia after the half and the standard dose. Animals infused with the double dose via either route exhibited significant K^+ decreases, resulting in moderate-to-severe hypokalemia with median potassium blood levels above 2.4 mEq/l (Figure 2.11 B).

In accordance with the previous experiment, BHB/M caused a dose-dependent increase in blood pH, which reached significance after infusion completion in animals receiving the double dose intravenously (Figure 2.11 C). Infusion of BHB/M resulted in moderate and moderate-to-severe alkalosis at the half and the standard or double dose, respectively.

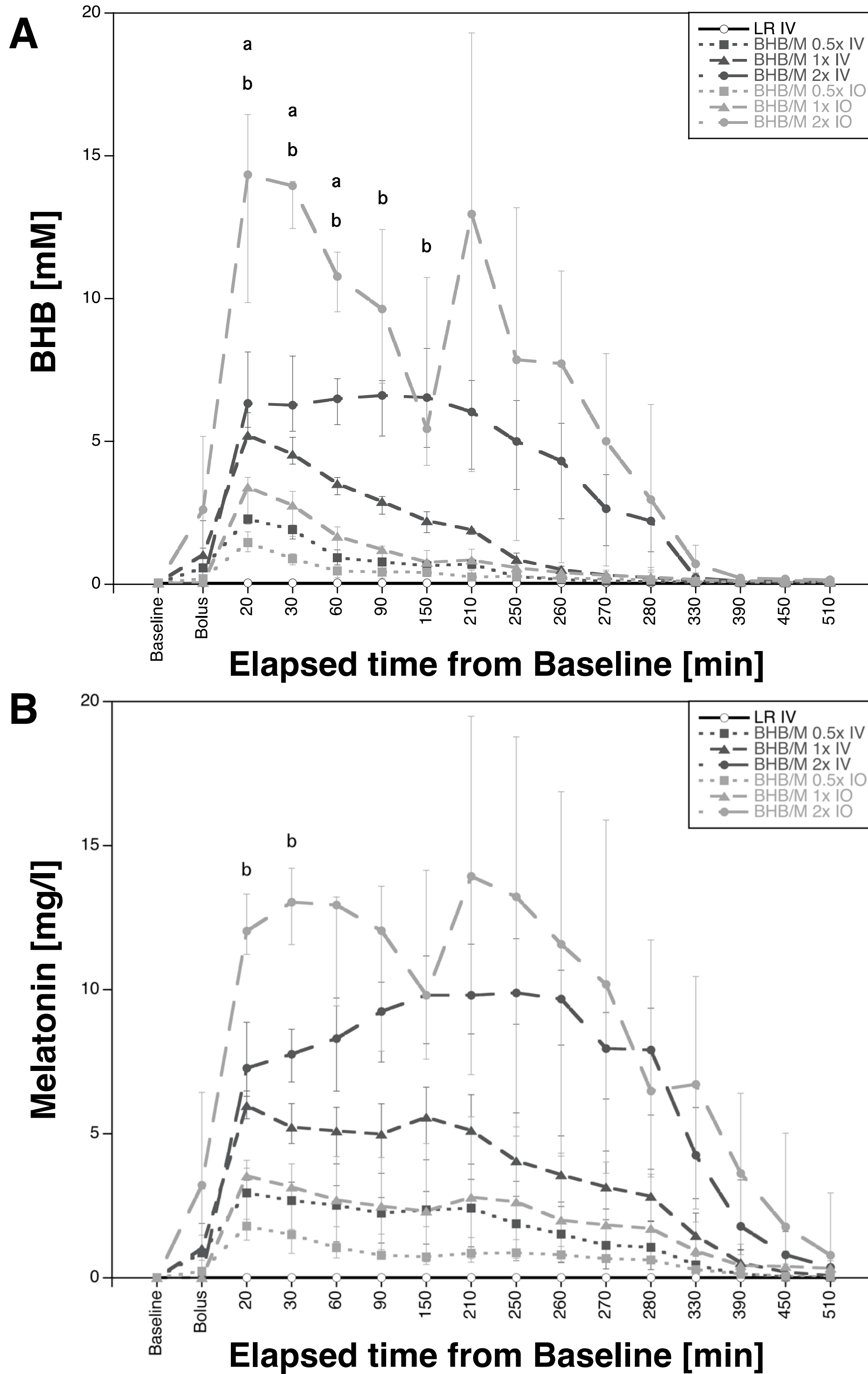

Figure 2.9. (A) BHB and (B) melatonin concentrations in healthy pigs exposed to different doses of BHB/M via intravenous or intraosseous infusion. Error bars depict IQR. N = 4 in all groups, except for BHB/M 2x IO group where n = 3. a p<0.05 BHB/M 0.5x IV – BHB/M 0.5x IO; b p<0.05 BHB/M 1x IV – BHB/M 1x IO.

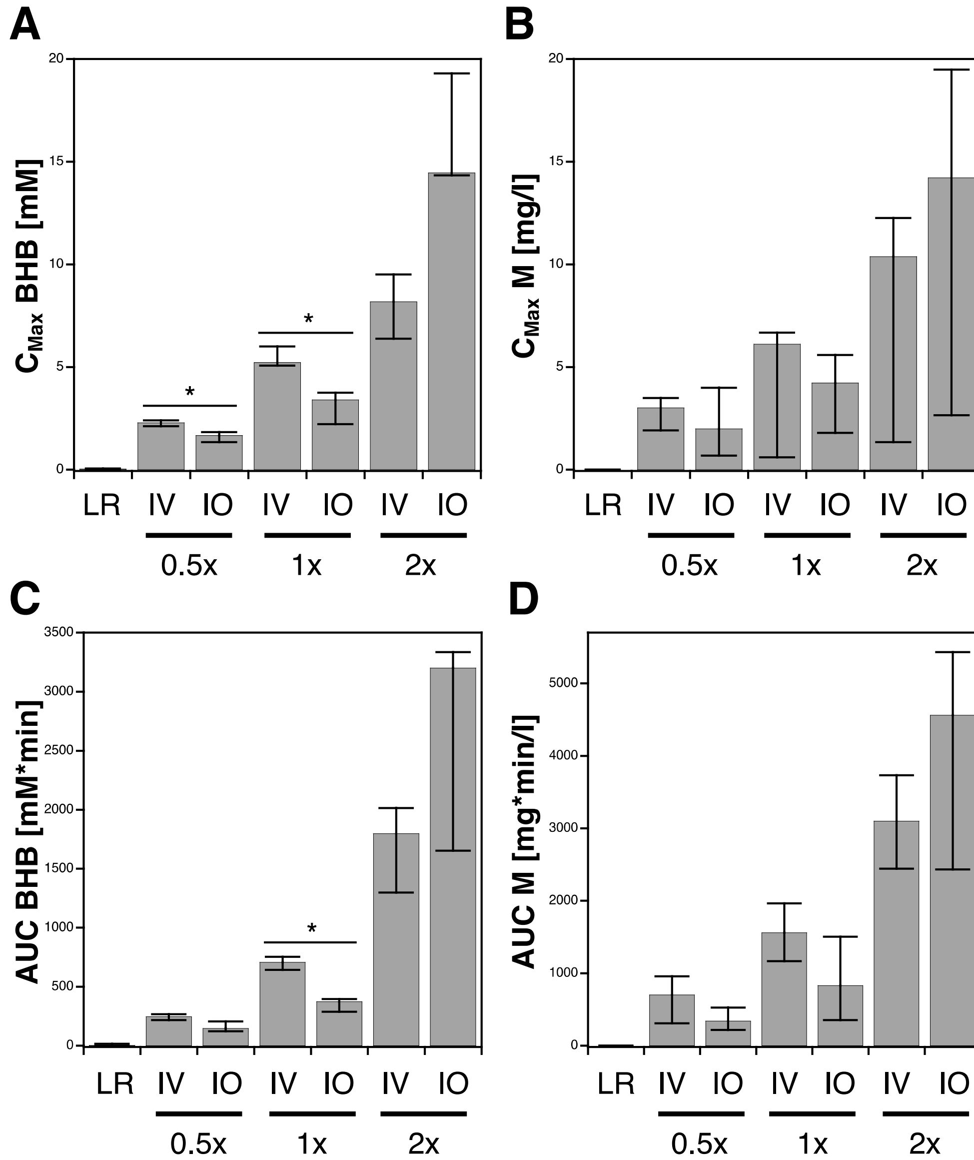

Figure 2.10. (A, B) Peak serum concentrations and (C, D) area under the curve (AUC) of D-ß-hydroxybutyrate and melatonin in healthy pigs exposed to different doses of BHB/M via intravenous or intraosseous infusion. Error bars depict IQR. n = 4 in all groups, except for 2x BHB/M IO group where n = 3. * $p < 0.05$ BHB/M IV – BHB/M IO for the respective dose.

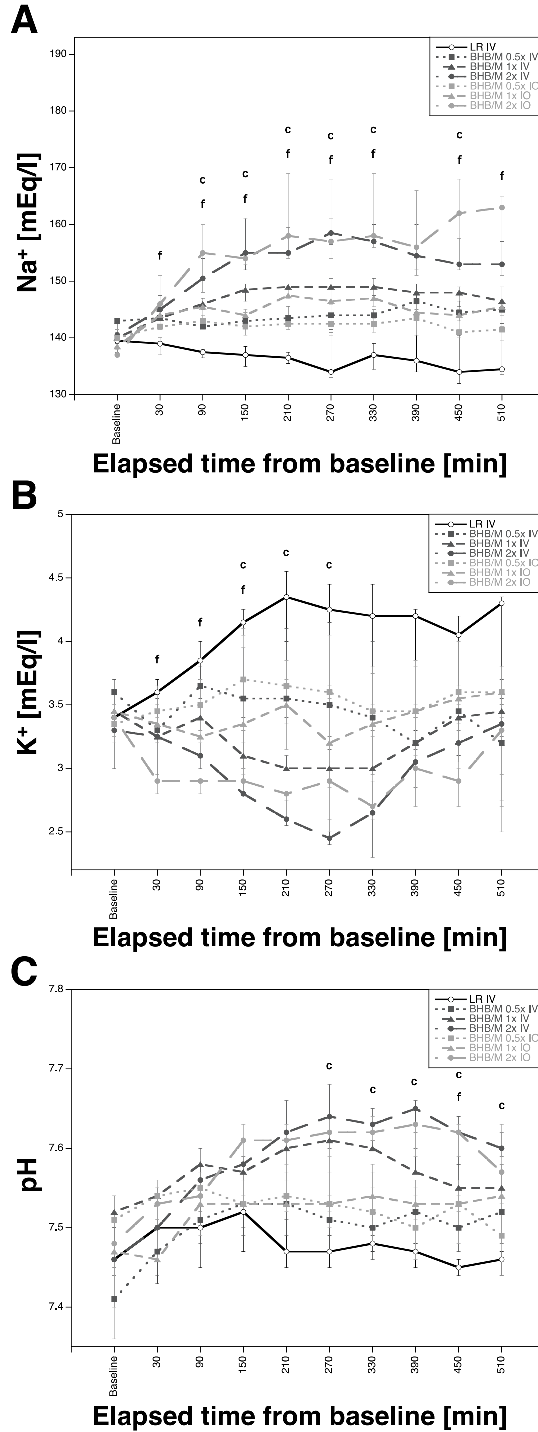

Figure 2.11. (A) Sodium and (B) potassium blood concentrations and (C) pH levels in healthy pigs exposed to different doses of BHB/M via intravenous or intraosseous infusion. Error bars depict IQR. N = 4 in all groups, except for BHB/M 2x IO where m = 3. c $p<0.05$ BHB/M 2x IV – LR IV; f $p<0.05$ BHB/M 2x IO – LR IV.

Discussion

This study was a continuation of our previous work in which we showed that BHB/M treatment was associated with increased survival in a pig model of hemorrhagic shock (232). Here, we omitted an osmotic or a DMSO vehicle group, as our previous study showed that a 4 M NaCl solution with 20% DMSO did not exert a therapeutic benefit (232). As this study focused on potential adverse drug effects, we compared the safety of BHB/M to standard of care treatment with LR. We introduced hemorrhagic shock with lung and liver injury as a more clinically relevant model to assess the safety of BHB/M. Furthermore; we evaluated the pharmacokinetic profile of BHB/M treatment in healthy pigs. Accounting for the acute nature of shock, we evaluated BHB/M via two routes of administration: intravenous and intraosseous infusion.

BHB/M administration did not result in significant changes in various physiological and hemodynamic measurements throughout the experiment (Table 2.2). We observed significantly lower hemoglobin levels in BHB/M-treated pigs at the end of the shock period. This may be a result of DMSO-induced hemolysis, which has previously been reported after injection of a solution containing 15% DMSO (263).

There were no significant differences between groups for various markers of organ damage throughout the experiment (Table 2.3). Serum total protein levels were higher in LR animals at two time points. However, we consider this observation not clinically relevant, as this was not an ongoing trend throughout the experiment. Surviving animals were followed for up to two weeks after treatment, during which no adverse biochemical or physiologic or safety concerns were detected. BHB/M did not negatively affect neurologic status, as there were no significant group differences in porcine Glasgow coma scores at different time points during follow-up. Furthermore, we did not observe any adverse effects that were not due to the experimental protocol when animals were examined via necropsy and histopathology. These findings suggest that BHB/M is a safe treatment for hemorrhagic shock with polytrauma in this large animal study.

As in our previously published study, lactate levels were higher in in BHB/M IV than in LR animals during early resuscitation (Table 2.2, Figure 2.3) (232). The reason for increased lactate levels may lie in the metabolic effect of BHB. Lactate is a marker of tissue hypoxemia and an active metabolite, and it can be used as an energy source for the brain at basal and especially at higher serum levels (271). Increased utilization of BHB

might lead to decreased lactate metabolism, resulting in raised lactate serum levels. Indeed, fasting-induced ketosis resulted in significantly increased brain lactate concentrations in humans, an effect that was attributed to the inhibitory effect of ketone body metabolism on lactate oxidation (272). Another reason for increased lactate levels may lie in the effect of alkalosis observed after BHB/M treatment. ATP is produced by the breakdown of glucose into pyruvate, which is converted to lactate during anaerobic conditions. The activity of phosphofructokinase 1 (PFK), a major regulatory enzyme in the conversion of glucose into pyruvate, is inhibited at low pH. Increased PFK-inhibition during alkalosis may result in increased pyruvate and consequently lactate production. Indeed, different studies have shown that lactate production is higher at increased blood pH (273). Consequently, correction of alkalosis during BHB/M treatment may result in decreased lactate levels.

BHB/M treatment resulted in significant increases in blood sodium levels, while potassium concentrations were significantly decreased after intravenous BHB/M infusion (Figure 2.4). Of note, all observed changes resolved within hours after infusion completion and were not associated with impaired organ function. In a recent study, comparable increases in sodium levels were observed for several days in patients resuscitated with hypertonic saline, without causing long-term adverse effects (274). Blood potassium levels were significantly lower in BHB/M IV than in LR-treated animals during the beginning of the resuscitation period. Thereafter, all three treatment groups experienced mild hypokalemia until the end of the resuscitation period. Severe hypokalemia can predispose to an increased risk for cardiac arrhythmias, which may result in cardiac arrest. However, arrhythmias are mainly observed in patients with underlying cardiac pathology (275). Importantly, hypokalemia did not result in severe cardiac adverse effects in this study, as all animals experiencing the most severe hypokalemia (BHB/M IV) survived until the end of the experiment. Although pigs were not treated to correct for hypokalemia, potassium levels returned to normal within 24 hours after the end of resuscitation (Figure 2.4 B).

All treatment groups experienced moderate alkalosis during the resuscitation phase, with highest pH levels in BHB/M IV animals during the first hours after shock (Figure 2.4 C). Metabolic alkalosis may lead to hypoventilation as an attempt to correct pH. As this study was conducted with anesthetized animals on a ventilator, we do not know

whether acute hypoventilation does occur as a result of BHB/M treatment. However, BHB/M IV treatment did not result in prolonged respiratory adverse effects, as (in contrast to LR treatment) no BHB/M pigs died after extubation. Hypokalemia is commonly associated with metabolic alkalosis and may potentiate the decrease of blood pH observed after BHB/M treatment. Consequently, correction of hypokalemia would likely decrease the alkalosis observed.

BHB/M IV treatment resulted in significantly increased urine output (Figure 2.5 A). This was not accompanied by an increase in pulmonary wedge pressure, mean pulmonary artery pressure, fluid administration or net fluids (Figure 2.2, Figure 2.5 B and data not shown). A potential reason for increased urine output in BHB/M IV pigs may be a diuretic effect of the administered drug. Indeed, a study in rhesus monkeys suggests that DMSO, which is part of the BHB/M formulation, exerts diuretic effects (276).

There was no significant difference in mortality between treatment groups (Figure 2.8). It is important to consider that this study was conducted to evaluate the safety of BHB/M via two routes of administration. As a result, this study was not powered to assess survival in this animal model, as the efficacy of the treatment was previously assessed in a rat and a porcine model of hemorrhagic shock (231, 232). The BHB/M IV group experienced the highest survival rate, while the BHB/M IO group had the fewest survivors. The described findings led us to hypothesize that the decreased survival after BHB/M IO infusion was due to BHB and melatonin serum levels below the therapeutic range. Indeed, BHB and melatonin serum levels were decreased in BHB/M IO pigs, along with less prominent changes in blood electrolytes and pH.

Pharmacokinetic dosing study

Drug serum concentrations

Intraosseous infusion is recommended in situations where intravenous access cannot be easily established, as it usually allows for rapid and reliable drug delivery (277). Interestingly, in this study, animals treated intravenously with the half or the standard dose of BHB/M experienced higher peak serum levels than those treated via the intraosseous route (Figure 2.9). This effect may be due to a slow distribution of the drug from the bone marrow to the systemic circulation. In a study by Jaimovich et al., phenytoin levels in the bone marrow 45 minutes after the end of infusion were higher

than the peak plasma levels observed during intraosseous administration (278). Interestingly, Tan and coworkers detected significantly higher melatonin levels in rat bone marrow than in serum, suggesting specific melatonin binding proteins in this tissue (279).

Paradoxically, when the double dose of BHB/M was administered, it resulted in higher C_{Max}, AUC and serum levels after intraosseous than after intravenous infusion for both BHB and melatonin (Fig. 2.9, Figure 2.10). Whether this effect is due to differences in drug protein binding, metabolism or distribution is not known and requires further investigation.

Physiology

The observed differences in drug serum concentrations were accompanied by variations in blood electrolytes and pH measurements (Figure 2.11). As in the efficacy experiment, these changes were transient and started to resolve with the end of infusion.

We did not observe significant group differences in various measurements of physiologic measurements, markers of organ function, or evidence of organ damage upon necropsy. Animals treated with BHB/M via either route appeared to be in good health after euthanasia even when the drug was administered at double the standard dose. These results confirm our finding that the administration of BHB/M in pigs is safe.

Limitations

The observed low mortality rate limits our study for the evaluation of treatment efficacy. However, the primary outcome of our research was not to evaluate BHB/M efficacy but to assess the safety of the treatment. Significant differences in survival may be observed in a model with a higher mortality rate in a larger study.

We hypothesized that the decreased survival after intraosseous BHB/M treatment was a result of sub-therapeutic serum levels of the administered compounds. Although intraosseous infusion in healthy pigs indeed resulted in lower drug serum levels after the half and the standard dose, we observed the opposite effect for the double dose. It is unclear what caused these discordant results. We did not test drug levels in the bone marrow, which would have been valuable for understanding the mechanism behind the variations in pharmacokinetics of intraosseous BHB/M. Furthermore, this study was conducted with a limited sample size (n=3-4 per group), which hinders definite

conclusions about the observed results. Future studies with higher animal numbers are necessary to clarify the effect of high intraosseous BHB/M doses on drug serum levels and to determine the impact of protein binding, metabolism, and drug distribution on BHB and melatonin serum levels after different routes of administration.

The experiments in this study were conducted after pigs were subjected to an overnight fast. Since fasting can affect ketone body serum levels and metabolism, it may influence serum concentrations and the effect of BHB during hemorrhagic shock (280). Further studies should evaluate BHB/M efficacy in both fasted and fed state.

Proposed mechanism of BHB/M

Despite existing studies on BHB and melatonin in hemorrhagic shock, the mechanism of action behind this combination is currently unknown. Hemorrhagic shock is characterized by decreased tissue perfusion, cellular oxygen, and energy supply, resulting in impaired mitochondrial respiration and ATP production (5, 8, 281). Paradoxically, when tissue perfusion is restored during resuscitation, rapid mitochondrial activation results in increased production of reactive oxygen species (ROS), oxidative stress and consequently cell and tissue damage (5, 8).

BHB is a ketone body and a physiological energy source (235, 282). Melatonin is an antioxidant and a radical scavenger (222). Existing data suggests that BHB and melatonin may exert their favorable effects by influencing cellular energy metabolism and oxidative stress (Figure 2.12).

A recent study utilizing a murine stroke model suggests that BHB exerts its neuroprotective effect via the hydroxyl-carboxylic acid receptor 2 (HCA_2) (235). In this study, BHB treatment significantly decreased infarct volume and neurological deficit in wild type, but not in *Hca2-/-* mice subjected to middle cerebral artery occlusion. The experiments suggest that this effect was likely mediated by prostaglandin released from infiltrating monocytes or macrophages (235). Further studies will have to be conducted to assess the role of HCA_2 activation in the beneficial BHB effects after hemorrhagic shock and polytrauma.

Administration of BHB stimulates mitochondrial respiration, resulting in increased oxygen consumption and ATP production (282-284). This effect is exerted even in the context of ischemia and decreased oxygen supply, as indicated by the observation that

pigs treated with BHB/M showed increased oxygen consumption during hemorrhagic shock (232). If BHB stimulates consistent mitochondrial respiration and ATP production during suboptimal oxygen supply, mitochondrial reactivation during resuscitation will be less prominent, resulting in decreased generation of oxidative stress. Indeed, ketone bodies significantly decreased markers of oxidative stress after ischemia (285).

Depletion of cellular antioxidant defense elements during ischemia makes cells particularly vulnerable to oxidative stress (240). Consequently, increasing cellular antioxidant levels may decrease reperfusion injury. Indeed, treatment with the antioxidant and radical scavenger melatonin has shown beneficial effects in animal models of hemorrhagic shock (222, 226). In addition to acting as an antioxidant itself, melatonin is also known to enhance the activity of endogenous antioxidants and antioxidant enzymes, including glutathione, superoxide dismutase and glutathione peroxidase (222). Furthermore, some of the metabolites generated via the reaction of melatonin with reactive oxygen species exhibit antioxidant activity themselves, thereby potentiating the effect of melatonin (222).

We hypothesize that, by inhibition of oxidative stress-induced mitochondrial damage, melatonin improves mitochondrial function during ischemia and reperfusion, thereby enhancing the effect of BHB. Studies suggest that melatonin increases mitochondrial activity during oxidative stress as well as under basal conditions, likely via stimulation of complexes of the mitochondrial electron transport chain (reviewed in (286)). Furthermore, melatonin treatment prevented shock-induced decrease in liver ATP levels and decreased markers of oxidative stress in rats subjected to trauma and hemorrhage (226). Future studies are necessary to evaluate the mechanism behind this novel treatment of hemorrhagic shock.

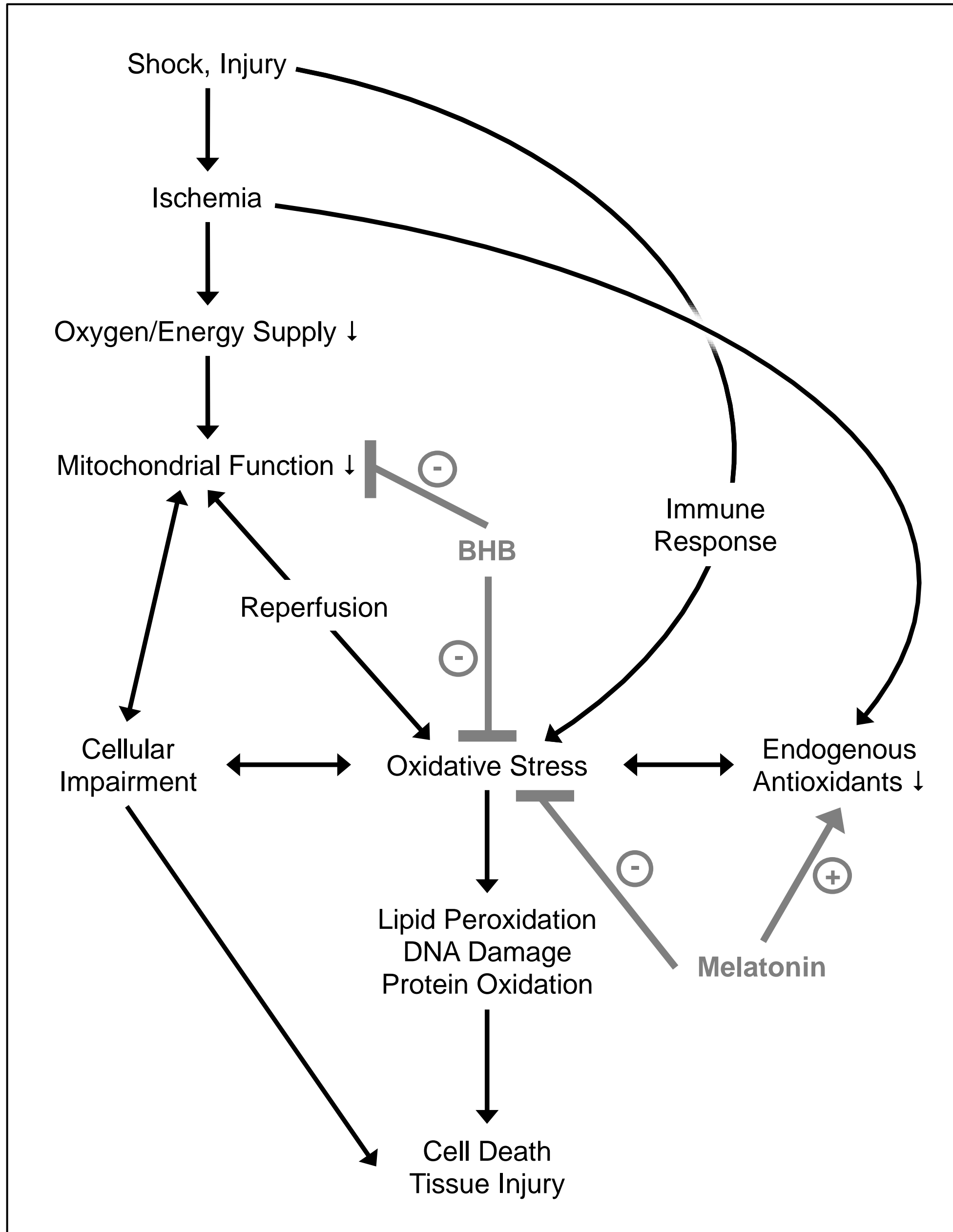

Figure 2.12. Proposed mechanism of action for D-β-hydroxybutyrate and melatonin in the treatment of hemorrhagic shock and injury. Limited tissue oxygen supply during severe blood loss and rapid mitochondrial activation upon reperfusion result in oxidative stress, decreased mitochondrial function and tissue injury. BHB improves mitochondrial respiration, thereby mitigating the induction of oxidative stress during reperfusion. Melatonin prevents oxidative-stress-induced cell damage via its direct antioxidant effect and by enhancing endogenous antioxidant systems. Furthermore, melatonin improves mitochondrial function directly, thereby decreasing reperfusion-induced oxidative stress and enhancing the effect of BHB.

Conclusions

In this study, we examined the safety of a D-β-hydroxybutyrate/melatonin low volume resuscitation fluid in a porcine model of hemorrhagic shock with polytrauma. Survival after treatment did not differ significantly between groups. Treatment with BHB/M did not result in serious adverse effects via intravenous or intraosseous infusion. Moderate, transient changes in blood electrolytes, pH and lactate levels were observed, all of which resolved at the end of resuscitation. Most importantly, we did not detect treatment-induced differences in physiological parameters, markers of organ function or histopathology for up to two weeks after the experiment. Our results suggest that 4 M BHB/43 mM melatonin in 20% DMSO is a safe treatment for severe blood loss with polytrauma in pigs.

Intraosseous BHB/M infusion of the standard dose resulted in decreased drug serum concentrations in injured and healthy pigs. This is likely due to BHB and melatonin retention in the bone marrow, rendering intravenous BHB/M the preferred route of administration at the standard dose. Lower serum concentrations after intraosseous infusion could be overcome by increasing the dose of BHB/M. The absence of treatment-induced differences in physiological parameters, organ function markers and histopathology in healthy pigs confirms our finding that infusion of BHB/M is safe.

We believe that the beneficial properties of BHB/M are due to a positive effect on mitochondrial respiration during ischemia, along with the inhibition of oxidative stress. This hypothesis will be tested in further studies.

Chapter 3. D-β-Hydroxybutyrate and Melatonin for Treatment of Porcine Hemorrhagic Shock and Injury: Determination of the Maximum Tolerated Dose and Evaluation of varying Melatonin Concentrations

Portions of this chapter were published in:

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Introduction

Infusion of a combination of 4 M D-β-hydroxybutyrate/43 mM melatonin (BHB/M) during early hemorrhagic shock significantly decreases mortality in preclinical rat and pig models (231, 232). The overarching goal of the work presented in this chapter was to optimize the BHB/M dose and concentration in regard to efficacy and safety of the treatment.

Our first objective was to define the maximum tolerated dose (MTD) of 4 M BHB/43 mM M in porcine hemorrhagic shock and polytrauma. The MTD, an important factor for subsequent toxicity studies, is defined as the highest dose of a drug that does not cause unacceptable toxicity (287). Determination of the no observed adverse effect level and the MTD in appropriate animal species lay the basis for phase 1 clinical trials, in which drug safety is evaluated to establish a recommended dose for phase 2 efficacy trials (288, 289). Animals often receive the MTD in long-term toxicity studies, as it increases the likelihood of identifying rare adverse events (290). Furthermore, by scaling for body surface area, the MTD can be used to normalize doses between species, which gives important information on the maximum safe dose in humans (291). We hypothesized that doubling the dose of BHB/M would not be associated with unacceptable adverse effects, while administration of four times the BHB/M standard dose would exert increased toxicity.

Our second objective was to test the efficacy of decreased melatonin concentrations in combination with 4 M BHB in porcine hemorrhagic shock, trauma and resuscitation. Due to the low aqueous solubility of melatonin, the 4 M BHB/43 mM M treatment solution contains 20% (v/v) dimethyl sulfoxide (DMSO). The parenteral use of DMSO is controversial (262), and DMSO has been associated with different adverse effects,

including emesis, anemia and intravascular hemolysis (246, 247, 263, 264). We therefore wanted to evaluate whether it would be possible to decrease the dose of melatonin, and hence DMSO, in the treatment without loss of efficacy. Decreased serum concentrations of BHB and melatonin after intraosseous infusion were associated with increased mortality (chapter 2). However, a later study showed that in rat hemorrhagic shock, the concentration of melatonin, but not BHB in the treatment could be decreased without loss of efficacy (233). Hence, the increase in mortality after intraosseous infusion may be a result of lowered BHB serum concentrations, while decreased levels of melatonin may still be effective. We hypothesized that solutions containing 4 M BHB and a melatonin concentrations of 43 mM would be equally as effective at improving post-hemorrhagic shock survival as solutions with 4 M BHB in combination with 20 mM, 10 mM, 4.3 mM, or 0.43 mM melatonin.

Materials and methods

Shock and resuscitation

All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol # 1306-30703A) and in accordance with the National Institutes of Health guidelines for ethical animal research. Yorkshire-Landrace pigs (15-25 kg, Manthei Hog Farm, LLC, Elk River, Minnesota) were exposed to our established shock and injury protocol (Figure 4.1). Pigs received ceftiofur antibiotic prophylaxis (5 mg/kg daily), and analgesia was administered during anesthesia (buprenorphine 0.03 mg/kg every 4 h) and after arousal (ketoprofen 2 mg/kg daily, buprenorphine 0.03 mg/kg twice daily). Induction of anesthesia, instrumentation, shock and injury, treatment infusion, limited resuscitation (R) and full resuscitation (FR), hemodynamic measurements, and analysis of blood gases, organ function markers and drug serum levels were conducted as described in chapter 2.

Surviving animals were extubated and recovered for 24 h, 48 h or 14 days. Animal care staff performed postoperative checks on wellness, body temperature, respiration and pulse at least twice daily. Pigs experiencing unrelieved pain or stress during recovery were sacrificed. Euthanasia was performed with beuthanasia solution (0.22 ml/kg intravenous).

Treatment infusion

Fifteen minutes after pulmonary contusion, during hemorrhage, pigs were infused with different solutions containing lactated Ringer’s solution (LR) or combinations of BHB and melatonin. Treatments were infused as a ten-minute bolus followed by four-hour continuous infusion of the respective fluid at a lower rate, as detailed in Table 3.1.

8-isoprostane ELISA

For 8-isoprostane analysis, urine samples were centrifuged (1000 RpM, 5 min), the supernatant was collected and mixed 1:1 with 1 M acetate buffer (pH 4). Samples were extracted using C-18 columns and quantified with ELISA (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s instructions. 8-isoprostane levels were normalized to urine output per body weight per hour (292). Samples collected at FR 7 h were used when material from FR 8 h was not available.

Statistical analysis

One pig in the 4x 4 M BHB/43 mM M group died during instrumentation prior to shock induction and was excluded from the analysis. A total of sixty-five (33 male, 32 female) pigs were analyzed in this set of experiments. Twenty-four of these pigs; 12 in the LR group, and 12 in the 1x 4 M BHB/43 mM M group were part of the experiment described in chapter 2. We then increased the numbers in both groups by adding additional animals, resulting in 16 pigs in the LR group and 14 pigs in the 1x 4 M BHB/43 mM M group, respectively.

Survival was analyzed via Kaplan-Meier analysis with generalized Wilcoxon test. Area under the curve (AUC) was calculated using PKSolver from baseline over five sampling time points ($AUC_{0\text{-}FR20}$) using the trapezoidal rule (270). Non-longitudinal data were analyzed via Kruskal-Wallis test with Dunn-Bonferroni corrections and are reported as medians with interquartile ranges (IQR). Longitudinal parameters were analyzed via Proc Mixed procedure in SAS Version 9.4 (SAS Institute, Inc., Cary, NC) and are depicted at key time points as least-squared means with 95% confidence intervals (CI). Group (G), Time (T) and Group*Time Interaction (G*T) were modeled as fixed effects. The models used compound symmetry or autoregressive covariance structure and the between-within method for degrees of freedom. For parameters with significant interaction effects, differences at individual time points were analyzed by pairwise comparisons with Tukey adjustments.

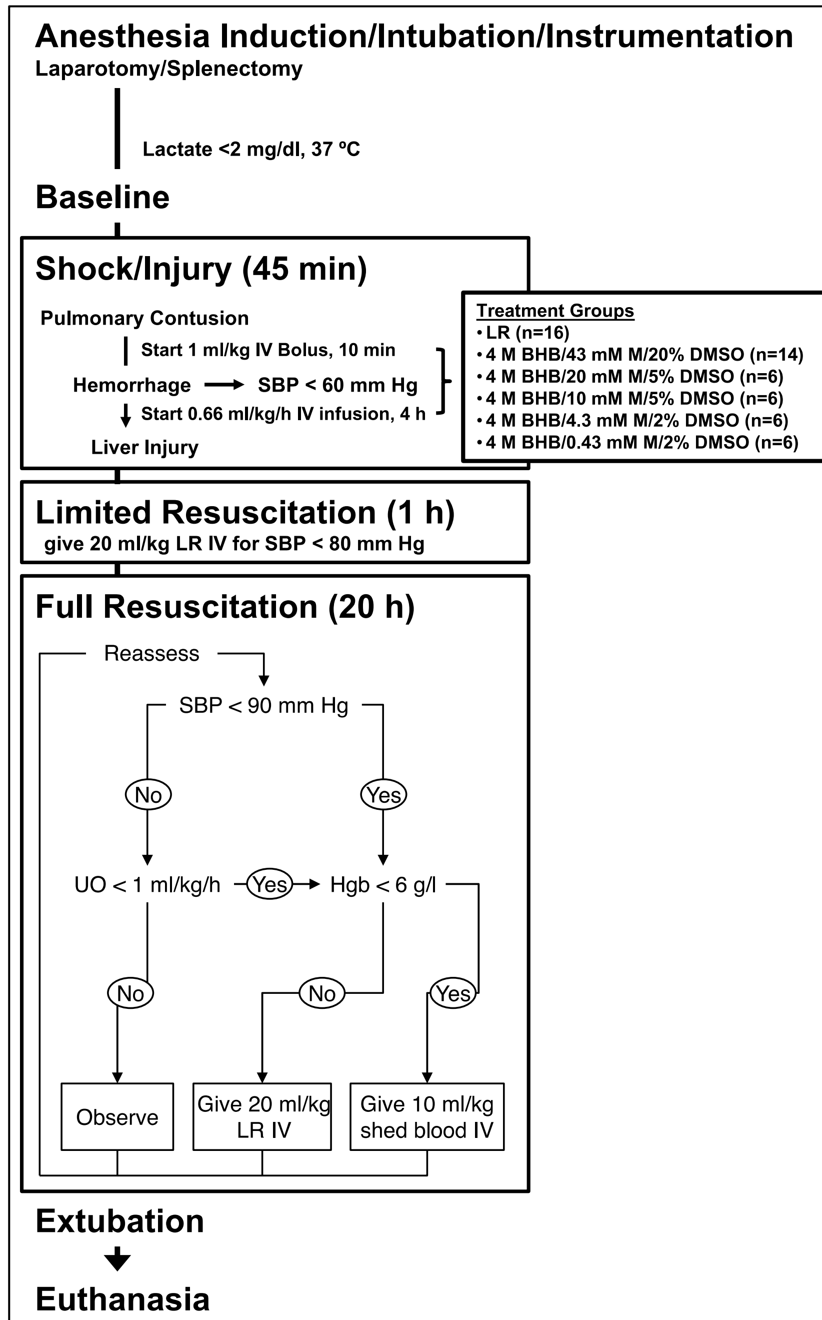


Figure 3.1. Shock, injury and resuscitation protocol. Pulmonary contusion was followed by blood withdrawal and creation of liver crush injuries with a Holcomb clamp (269). Fifteen minutes after contusion, treatment solutions were administered as a 1 ml/kg bolus, immediately followed by 0.66 ml/kg/h continuous infusion over 4 hours (3.64 ml/kg total). Pigs received limited resuscitation, throughout which they were evaluated every ten minutes and, if necessary, received boluses of LR. An hour later, full resuscitation was initiated, throughout which pigs received intravenous LR and shed blood. Modified from (293).

Table 3.1. Treatment groups evaluated in chapter 3.

<table>
<tr><th>Treatment</th><th>[Mel] mM</th><th>Total Melatonin Dose</th><th>[BHB] M</th><th>Total BHB dose</th><th>% DMSO</th><th>10 min Bolus</th><th>4 h Continuous Infusion</th><th>N</th></tr>
<tr><td>LR</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>16</td></tr>
<tr><td>1x 4 M BHB/43 mM M</td><td>43</td><td>156.62 µmol/kg</td><td rowspan="7">4</td><td>14.56 mmol/kg</td><td>20</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>14</td></tr>
<tr><td>2x 4 M BHB/43 mM M</td><td>43</td><td>313.04 µmol/kg</td><td>29.12 mmol/kg</td><td>20</td><td>2 ml/kg</td><td>1.32 ml/kg/h</td><td>6</td></tr>
<tr><td>4x 4 M BHB/43 mM M</td><td>43</td><td>626.08 µmol/kg</td><td>58.24 mmol/kg</td><td>20</td><td>4 ml/kg</td><td>2.64 ml/kg/h</td><td>5</td></tr>
<tr><td>4 M BHB/20 mM M</td><td>20</td><td>72.8 µmol/kg</td><td rowspan="4">14.56 mmol/kg</td><td>5</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>6</td></tr>
<tr><td>4 M BHB/10 mM M</td><td>10</td><td>36.4 µmol/kg</td><td>5</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>6</td></tr>
<tr><td>4 M BHB/4.3 mM M</td><td>4.3</td><td>15.66 µmol/kg</td><td>2</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>6</td></tr>
<tr><td>4 M BHB/0.43 mM M</td><td>0.43</td><td>1.57 µmol/kg</td><td>2</td><td>1 ml/kg</td><td>0.66 ml/kg/h</td><td>6</td></tr>
</table>

Objective 1: D-β-Hydroxybutyrate and Melatonin for the Treatment of Porcine Hemorrhagic Shock and Injury: Determination of the Maximum Tolerated Dose.

Results

Shock induction, resuscitation and hemodynamics

We examined the effects of administration of 2x 4 M BHB/43 mM M and 4x 4 M BHB/43 mM M in our porcine model of hemorrhagic shock and polytrauma. There was no difference in shock induction, as the amount of blood withdrawn per kg bodyweight did not differ significantly between groups ($p = 0.81$). In accordance with the experimental protocol, pigs were resuscitated with boluses of LR (limited resuscitation phase) and LR or shed blood (full resuscitation phase) to meet endpoints of systolic blood pressure, urine output and hemoglobin (Figure 3.1). The cumulative net fluid balance indicates dose-dependent increases in fluid requirements after treatment with 2x 4 M BHB/43 mM M and 4x 4 M BHB/43 mM M (Figure 3.2 A). While BHB/M treatment was accompanied by elevated urine output in the 1x and 2x 4 M BHB/43 mM M group, pigs receiving the highest volume of 4 M BHB/43 mM M experienced decreased fluid excretion (Figure 3.2 B). As expected, shock induction caused a prominent decrease in mean arterial pressure (MAP) (Figure 3.2 C). MAP recovered during resuscitation, but was lowest in the 2x 4 M BHB/43 mM M and the 4x 4 M BHB/43 mM M group throughout resuscitation. Cardiac output dropped and heart rate increased during hemorrhagic shock and both parameters recovered during resuscitation (Table 3.2).

Survival

Forty-eight hours after the end of resuscitation, there was a significant difference in overall survival between groups ($p < 0.0001$). Survival was not significantly different between the 4 M BHB/43 mM M (13/14) and the LR group (10/16, $p = 0.081$). Significantly more pigs survived in the 4 M BHB/43 mM M than in the 2x 4 M BHB/43 mM M group (2/6, $p = 0.01$). All animals treated with 4x 4 M BHB/43 mM M succumbed within 17 hours after the end of shock, and survival in this group (0/5) was significantly lower than in all other treatment groups ($p \leqq 0.009$).

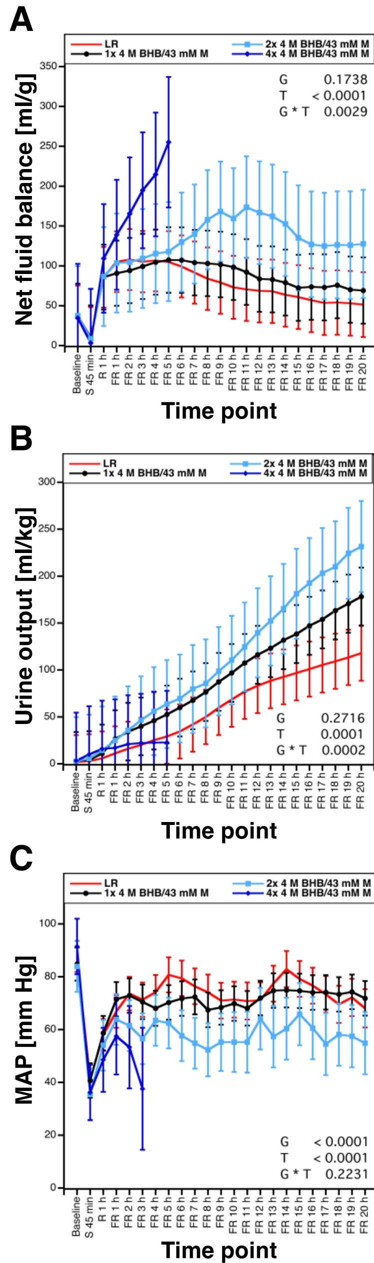


Figure 3.2. (A) Cumulative net fluid balance, (B) cumulative urine output and (C) mean arterial pressure in pigs exposed to hemorrhagic shock, polytrauma and resuscitation and treated with LR or different volumes of 4 M BHB/43 mM M. Cumulative net fluids [(LR infused + blood infused – blood withdrawn - urine output)/weight] and cumulative urine output [urine output/weight] are shown as the sum of all previous and the current time point (e.g. cumulative net fluids at R 1h = net fluids $_{baseline}$ + net fluids $_{S\ 45\ min}$ + net fluids $_{R\ 1\ h}$).

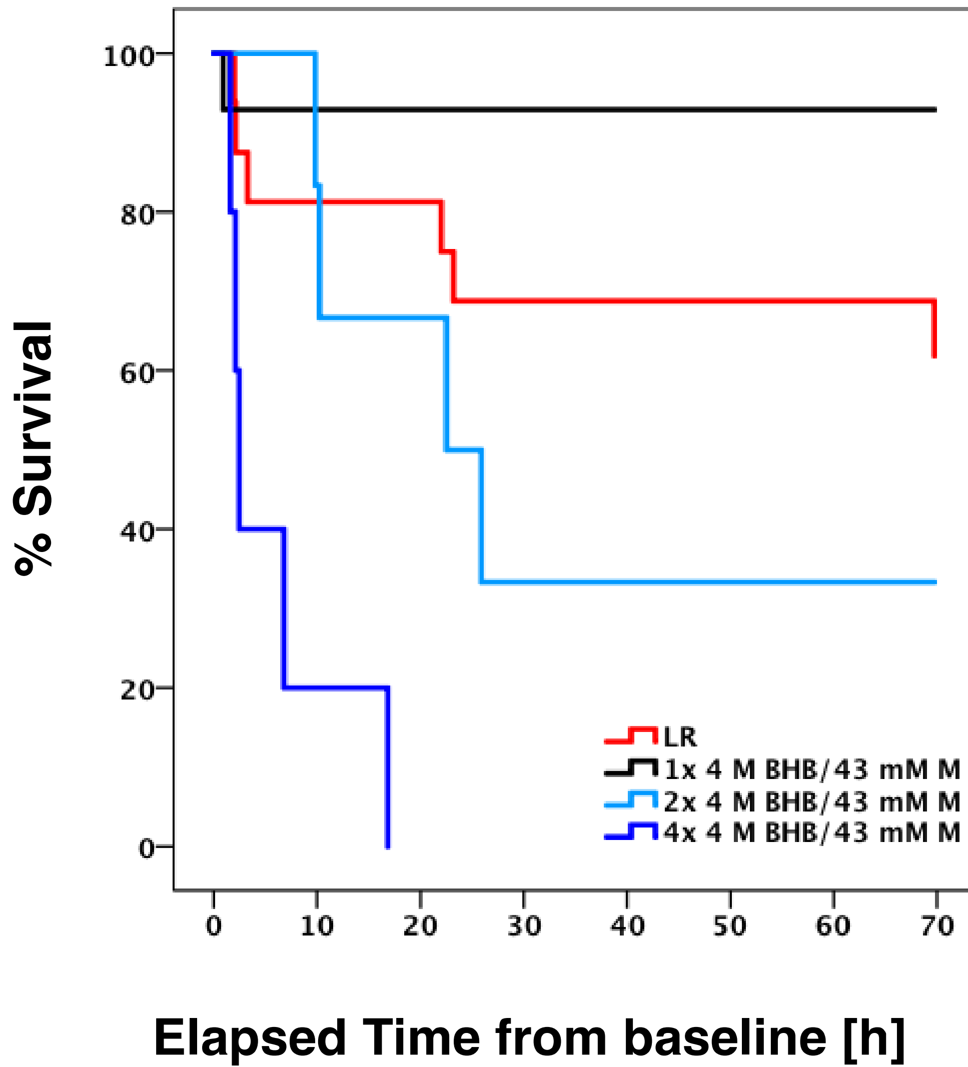


Figure 3.3. Survival of pigs exposed to polytrauma, hemorrhagic shock and resuscitation and treated with LR or different volumes of 4 M BHB/43 mM M.

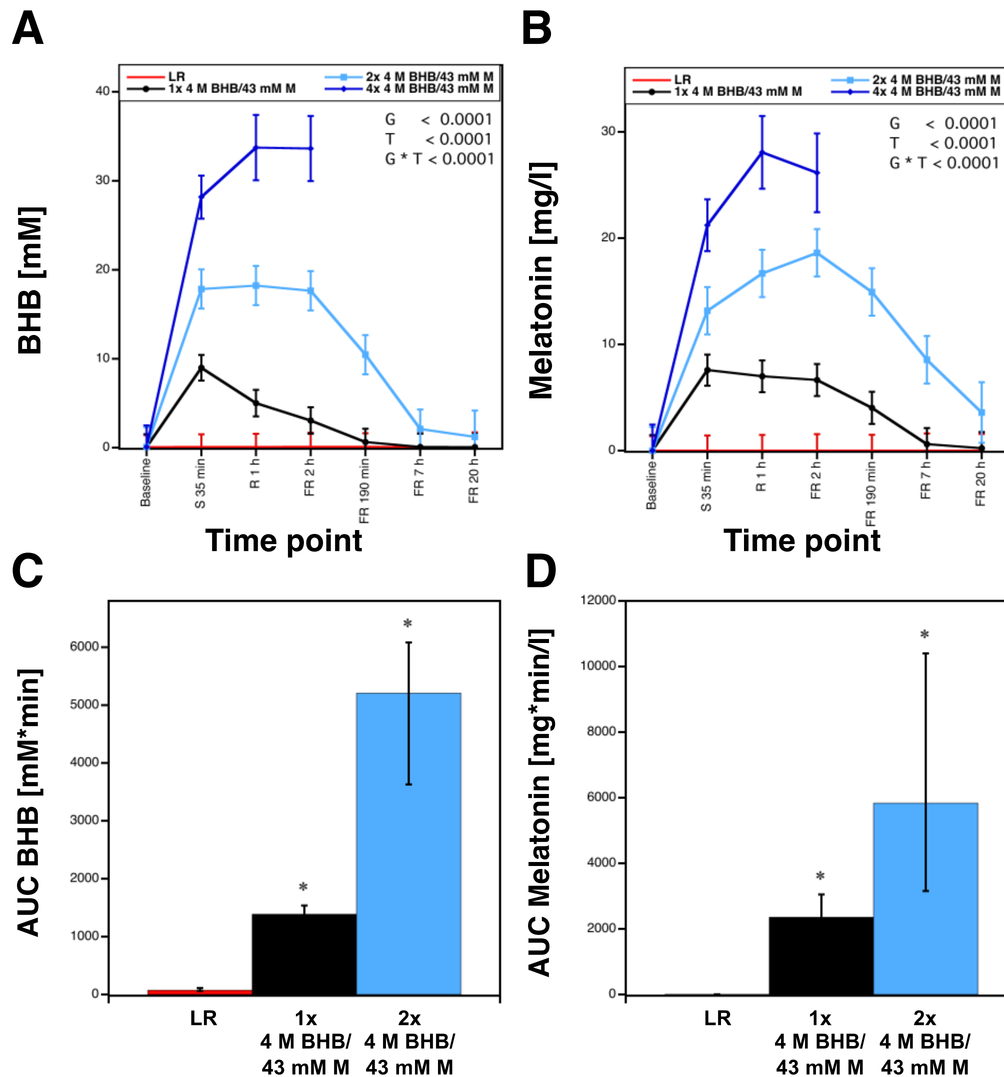


Figure 3.4. (A, B) Serum concentrations and (C, D) areas under the curve of BHB and melatonin in pigs exposed to polytrauma, hemorrhagic shock and resuscitation and treated with LR or different volumes of 4 M BHB/43 mM M. Area under the curve was only calculated for animals with serum concentrations measured until FR 20 h. As all pigs in the 4x 4 M BHB/43 mM M group succumbed before the end of resuscitation, AUC were not calculated for this group. * p<0.05 versus LR.

Drug serum levels

Serum levels of both BHB and melatonin increased in pigs treated with different doses of 4 M BHB/43 mM M in a dose-dependent fashion and peaked at the end of the limited resuscitation phase in all 4 M BHB/43 mM M treated groups (Figure 3.4 A, B). Drug serum concentrations were significantly different between all four groups during shock and at the end of the limited resuscitation phase. Drug levels were not significantly different between the 1x 4 M BHB/43 mM M and the control group thereafter but remained elevated in the 2x 4 M BHB/43 mM M and the 4x 4 M BHB/43 mM M groups. We did not observe changes in melatonin levels and only minimal increases in BHB concentrations in the LR group. Drug exposure over time (AUC) dose-dependently increased with BHB/M infusion for both BHB and melatonin (Figure 3.4 C, D).

Serum electrolytes, blood gases and markers of organ function

Pigs treated with BHB/M experienced dose-dependent, significant increases in serum sodium levels (Figure 3.5 A). Potassium levels were significantly higher in LR-treated pigs than in those receiving BHB/M at the end of the shock period, but there was no prominent dose-dependent effect of BHB/M (Table 3.2). Infusion of high volumes of 4 M BHB/43 mM M was associated with raised markers of shock severity and organ injury. While all treatment groups experienced shock-induced increases in systemic lactate levels, they were most prominent in the 2x 4 M BHB/43 mM M and the 4x 4 M BHB/43 mM M-treated groups (Figure 3.5 B). BHB/M-infusion increased pH in the standard and double-dose groups; however, pigs receiving 4x 4 M BHB/43 mM M experienced a drop in pH and moderate acidosis from shock until death (Figure 3.5 C).

Pigs receiving 4x 4 M BHB/43 mM M experienced sharp increases in bladder pressure, mean pulmonary artery pressure and pulmonary wedge pressure from the beginning of resuscitation until death (Figure 3.6, Table 3.2). There was a significant group effect for PaO_2:FiO_2 ratios, which were lowest in the 2x and 4x 4 M BHB/43 mM M treatment groups.

We observed significant interaction effects for hemoglobin, levels of aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) (Table 3.2). As intended, hemoglobin dropped after the onset of shock in all groups. Hemoglobin levels increased in the 4x 4 M BHB/43 mM M group during early resuscitation, resulting in concentrations close to baseline levels in this group.

Hemorrhagic shock and polytrauma caused progressive rises in CK and LDH as well as increased serum AST concentrations, which peaked during early resuscitation. Increases of these markers were more prominent with elevated 4 M BHB/43 mM M doses. Serum LDH concentrations were significantly higher in 2x 4 M BHB/43 mM M animals than in the other treatment groups after 4 and 8 hours of full resuscitation, and significantly higher in 2x 4 M BHB/43 mM M than in LR-treated pigs after 16 and 20 hours of full resuscitation. AST serum levels were highest in 4x 4 M BHB/43 mM M animals right before death, and significantly higher in 2x 4 M BHB/43 mM M than in LR or 1x 4 M BHB/43 mM M–infused animals after 4 and 8 hours of full resuscitation. There were no obvious treatment-dependent effects on body temperature, oxygen consumption, serum levels of alanine aminotransferase, albumin, bilirubin, blood urea nitrogen and alkaline phosphatase (not shown).

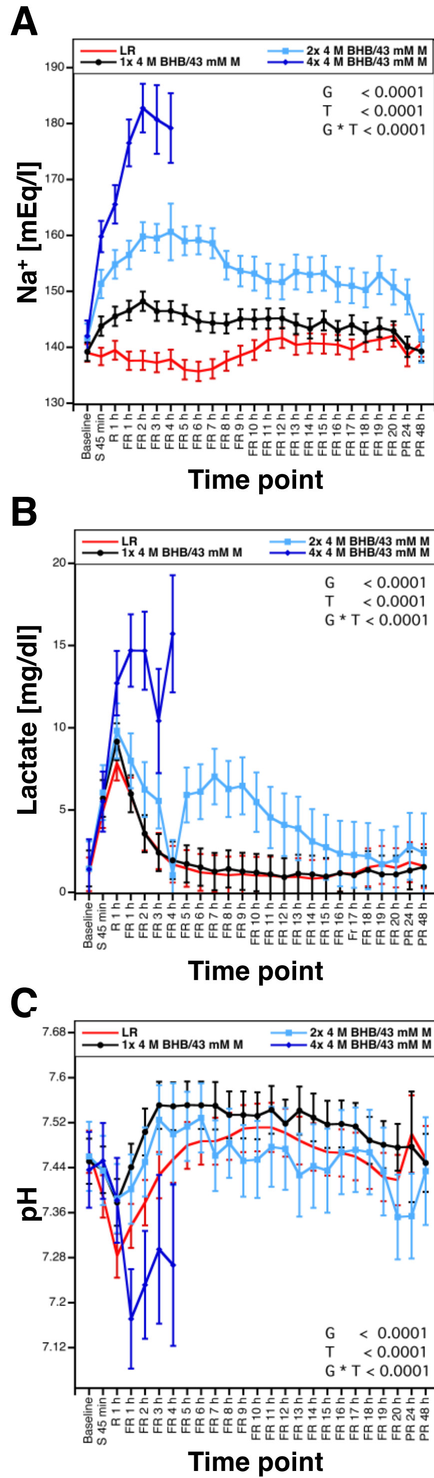


Figure 3.5. Serum levels of (A) Na^+, (B) lactate and (C) arterial blood pH in pigs exposed to polytrauma, hemorrhagic shock and resuscitation and treated with LR or different volumes of 4 M BHB/43 mM M.

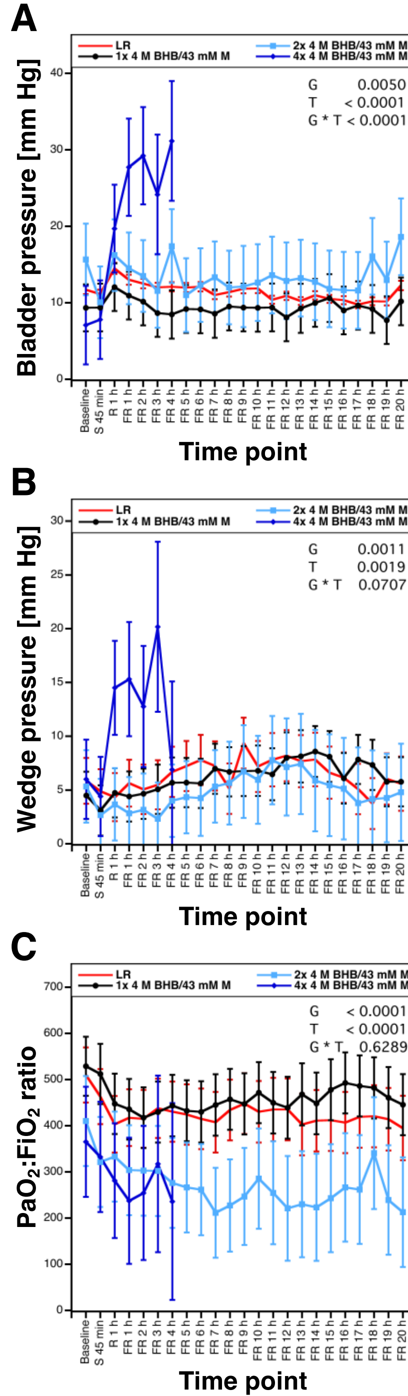


Figure 3.6. (A) Bladder pressure, (B) wedge pressure and (C) PaO_2:FiO_2 ratios in pigs exposed to hemorrhagic shock, polytrauma and resuscitation and treated with LR or different volumes of 4 M BHB/43 mM M. The PaO_2:FiO_2 ratio is the ratio of arterial atrial pressure of oxygen (PaO_2) to the fraction of inspired oxygen (FiO_2).

Table 3.2. Physiologic parameters and markers of organ function in pigs exposed to hemorrhagic shock, injury and resuscitation.

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 48 h
Heart Rate [beats/ min]			Effects: G .0076 T <.0001 G*T .0025			
LR	120 (1030-0136)	237 (221 - 254)	176 (159 – 192)	169 (152 – 187)	158 (139 – 178)	
1x 4 M BHB/43 mM M	125 (108 – 142)	198 (180 – 216)	179 (161 – 197)	151 (133 – 169)	144 (126 – 146)	
2x 4 M BHB/43 mM M	118 (91 – 144)	231 (204 – 257)	211 (184 – 137)	210 (183 – 236)	139 (106 – 171)	
4x 4 M BHB/43 mM M	132 (103 – 161)	246 (217 – 275)	170 (136 – 204)	208 (165 – 202)		
Cardiac Output [l/min]			Effects: G .0256 T <.0001 G*T .0016			
LR	2.9 (2.4 – 3.4)	1.3 (0.8 – 1.8)	2.9 (2.4 – 3.5)	4.4 (3.9 – 5.0)	4.3 (3.7 – 4.9)	
1x 4 M BHB/43 mM M	2.9 (2.4 – 3.5)	1.6 (1.0 – 2.3)	4.1 (3.5 – 4.7)	5.1 (4.5 – 5.6)	4.0 (3.5 – 4.6)	
2x 4 M BHB/43 mM M	3.2 (2.4 – 4.0)	1.5 (0.7 – 2.3)	2.7 (1.8 – 3.5)	3.9 (3.0 – 4.7)	2.9 (1.9 – 3.9)	
4x 4 M BHB/43 mM M	3.4 (2.5 – 4.3)	2.2 (1.3 – 3.1)	1.4 (0.2 – 2.6)	4.2 (2.8 – 5.5)		
MPAP [mm Hg]			Effects: G .0047 T <.0001 G*T .0016			
LR	19.9 (16.9 – 22.)	16.2 (13.2 – 19.2)	26.3 (23.2 – 29.4)	25.6 (22.3 – 28.8)	19.0 (15.3 – 22.6)	
1x 4 M BHB/43 mM M	17.3 (14.1 – 20.5)	14.1 (10.8 – 17.4)	25.2 (21.9 – 28.5)	18.6 (15.3 – 21.9)[•]	17.9 (14.6 – 21.3)	
2x 4 M BHB/43 mM M	22.2 (17.3 – 27.1)	14.5 (9.6 – 19.4)	20.1 (15.2 – 24.9)	19.0 (14.1 – 23.9)	21.3 (15.3 – 27.2)	
4x 4 M BHB/43 mM M	18.9 (13.5 – 24.2)	14.0 (8.7 – 19.3)	31.1 (24.8 – 37.4)	38.6 (30.5 – 46.8)[#]		

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 48 h
K^+ [mEq/l]			Effects: G <.0001 T <.0001 G*T <.0001			
LR	3.9 (3.7 – 4.2)	5.8 (5.6 – 6.1)[#▲●]	3.9 (3.7 – 4.2)[#]	4.3 (4.1 – 4.6)[#▲]	3.3 (3.0 – 3.6)	3.3 (2.9 – 3.6)
1x 4 M BHB/43 mM M	4.0 (3.7 – 4.2)	4.6 (4.3 – 4.9)[*]	3.0 (2.7 – 3.3)[*]	3.2 (2.9 – 3.4)[*]	3.3 (3.0 – 3.5)	3.5 (3.2 – 3.8)
2x 4 M BHB/43 mM M	3.7 (3.3 – 4.1)	4.3 (3.9 – 4.7)[*]	3.0 (2.6 – 3.4)	2.9 (2.5 – 3.3)[*]	4.0 (3.5 – 4.5)	3.6 (3.0 – 4.3)
4x 4 M BHB/43 mM M	3.9 (3.5 – 4.3)	4.2 (3.7 – 4.6)[*]	3.5 (2.9 – 4.0)	2.9 (2.2 – 3.6)		
Hemoglobin [g/dl]			Effects: G .0238 T <.0001 G*T <.0001			
LR	8.7 (8.4 – 9.0)	7.0 (6.6 – 7.3)	5.2 (4.8 – 5.5)	6.1 (5.7 – 6.4)	5.7 (5.3 – 6.1)	7.0 (6.5 – 7.5)
1x 4 M BHB/43 mM M	8.6 (8.2 – 8.9)	6.1 (5.7 – 6.4)	4.9 (4.6 – 5.3)	6.0 (5.7 – 6.4)	5.6 (5.3 – 6.0)	6.4 (6.1 – 6.8)
2x 4 M BHB/43 mM M	8.4 (7.8 – 9.0)	5.8 (5.2 – 6.4)	5.5 (4.8 – 6.1)	6.1 (5.6 – 6.7)	5.8 (5.0 – 6.7)	6.5 (5.6 – 7.4)
4x 4 M BHB/43 mM M	8.1 (7.6 – 8.7)	5.5 (4.9 – 6.1)	4.5 (3.7 – 5.3)	8.0 (7.1 – 8.9)		
AST [U/dl]			Effects: G .0473 T <.0001 G*T <.0001			
LR	31 (-216 – 278)	111 (-137 – 358)	143 (-104 – 390)	177 (-96 – 401)	112 (-160 – 385)	149 (-136 – 435)
1x 4 M BHB/43 mM M	32 (-232 – 296)	84 (-182 – 350)	148 (-119 – 415)	196 (-71 – 464)	136 (-136 – 409)	73 (-201 – 347)
2x 4 M BHB/43 mM M	34 (-369 – 437)	140 (-263 – 543)	321 (-82 – 724)	663 (259 – 1066)	733 (272 – 1193)	637 (139 – 1135)
4x 4 M BHB/43 mM M	36 (-406 – 478)	100 (-342 – 541)	168 (-290 – 627)	435 (-57 – 927)		

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 48 h
CK [U/dl]			Effects: G .4081 T <.0001 G*T .0111			
LR	475 (-780 – 1730)	679 (-586 – 1945)	888 (-379 – 2155)	1761 (429 – 3094)	3230 (1767 – 4693)	4860 (3195 – 6524)
1x 4 M BHB/43 mM M	463 (-879 – 1804)	627 (-747 – 2001)	868 (-521 – 2257)	2089 (704 – 3475)	3734 (2342 – 5126)	2937 (1517 (4358)
2x 4 M BHB/43 mM M	532 (-1517 – 2581)	616 (-1434 – 2665)	1125(-924 – 3174)	1685 (-364 – 3770)	7266 (4761 – 9771)	4756 (1692 – 7819)
4x 4 M BHB/43 mM M	612 (-1633 – 2857)	613 (-1632 – 2858)	747 (-1859 -3352)	2296 (-761 – 5353)		
LDH [U/dl]			Effects: G .0041 T <.0001 G*T <.0001			
LR	1122 (371 – 1872)	1315 (563 – 2068)	1406 (653 – 2159)	1882 (1116 – 2649)	2800 (1963 – 3637)[▲]	3544 (2659 – 4430)
1x 4 M BHB/43 mM M	1098 (296 – 1901)	1166 (356 – 1976)	1443 (630 – 2256)	2429 (1614 – 3244)	3062 (2233 – 3892)	3266 (2430 – 4102)
2x 4 M BHB/43 mM M	1144 (-82 – 2369)	1349 (123 – 2574)	2246 (1020 – 3471)	3860 (2634 – 5086)	6194 (4769 – 7618)[*]	5707 (4146 - 7268)
4x 4 M BHB/43 mM M	1309 (-33 – 2652)	1296 (-47 – 2638)	1414 (6 – 2822)	3322 (1789 – 4855)		

Data are presented as least-squared means (95% confidence interval). [#] p<0.05 vs 4 M BHB/43 mM M, [▲] p<0.05 vs 2x 4 M BHB/43 mM M, [•] p<0.05 vs 4x 4 M BHB/43 mM M.

Discussion

Here, as outlined in objective 1, we evaluated the safety and efficacy of increased volumes of 4 M BHB/43 mM M in our porcine model of hemorrhagic shock, trauma and resuscitation. We hypothesized that doubling the volume of 4 M BHB/43 mM M would not be associated with unacceptable adverse effects, while administration of four times the 4 M BHB/43 mM M standard dose would exert increased toxicity. Pigs treated with 4x 4 M BHB/43 mM M experienced significantly higher mortality (100% within 17 hours after shock) than pigs receiving 1x 4 M BHB/43 mM M, 2x 4 M BHB/43 mM M or LR ($p \leqq 0.009$). Mortality was also higher in pigs receiving 2x 4 M BHB/43 mM M than those treated with 1x 4 M BHB/43 mM M ($p = 0.01$). The increased number of deaths is likely a result of 4 M BHB/43 mM M-induced hypernatremia and increased intraabdominal pressure due to fluid translocation into the interstitial space.

Hypernatremia is common in the intensive care setting (294-297), and it has been associated with increased ventilator days, length of stay, renal dysfunction and mortality (297-301). Adverse effects of increased blood sodium levels include muscle cramps, seizures, arrhythmias, lethargy and coma (302-304). Patients with severe hypernatremia after treatment with 3% hypertonic saline for brain injury experienced increased markers of kidney injury; however, this did not result in renal failure (305).

In acute hypernatremia, increased plasma osmolality leads to a fluid shift from cells into the extracellular fluid, resulting in cell shrinkage (306, 307). Cell shrinkage is tolerated in most organs; however, it can cause cerebral hemorrhage and the rupture of meningeal vessels, resulting in neurological dysfunction or death (302, 308-312). Furthermore, rapid reversal of hypernatremia can lead to pronounced cerebral edema, although this is more common after chronic hypernatremia (313, 314).

BHB is administered as Na-D-β-hydroxybutyrate and, unsurprisingly, infusion of 4 M BHB/43 mM M was associated with dose-dependent increases in serum sodium concentrations (Figure 3.5). Pigs receiving intravenous 1x 4 M BHB/43 mM M experienced average peak levels of ~148 mEq/l Na^+ (Figure 3.5). Mild hypernatremia, defined as a serum sodium concentration of 145-150 mEq/l, was not associated with increased mortality in patients with traumatic brain injury (315). Patients treated with 5% hypertonic saline experienced mild hypernatremia (~150 mg/dl Na^+) with elevated sodium levels for 3 days without obvious adverse effects or increased mortality (274). In patients in the neurologic/neurosurgical intensive care unit, only severe hypernatremia

(>160 mEq/l Na^+) was independently associated with increased mortality (298). Animals receiving 2x 4 M BHB/43 mM M experienced severe hypernatremia, with an average peak ~161 mEq/l Na^+, which returned to baseline levels within 48 hours after the end of resuscitation. It should be noted that in our previous experiments, uninjured pigs receiving up to 2x 4 M BHB/43 mM M remained in good health despite mild-to-moderate hypernatremia (Chapter 2). This indicates that mild-to moderate hypernatremia alone was not lethal in pigs, but likely exacerbated trauma-associated adverse effects. We observed drastic increases in blood sodium levels in animals receiving 4x 4 M BHB/43 mM M, with peak levels greatly exceeding critical values (~183 mEq/l Na^+). These animals received a total of 58.24 mmol Na^+/kg over 4 hours. In a 70 kg adult, this would be equivalent to over 4 moles, or almost 92 g of sodium. For comparison, the recommended daily sodium intake is 2.3 g (316). Sodium was further increased by the administration of LR for resuscitation, as 1 l of LR contains ~2.4 g sodium. Cases in which sodium levels exceed 180 mEq/l generally result in death (317), as did hypernatremia associated with infusion of 4x 4 M BHB/43 mM M.

In addition to the induction of hypernatremia, our results suggest that infusion of high 4 M BHB/43 mM M doses was associated with increased fluid translocation into the interstitial space. One of the pathophysiologic responses to hemorrhagic shock is the loss of the integrity of the endothelium of the microvasculature, which is magnified by trauma-induced release of inflammatory mediators (318-320). When the integrity of this barrier is impaired, increased capillary permeability leads to a loss of proteins and electrolytes from the vasculature into the interstitial space (321). The accompanying decrease in oncotic pressure results in the translocation of extracellular fluid volume into the interstitial space, known as "third-spacing" (322-324). Third-spacing likely occurred to some extend in all experimental groups. However, 4 M BHB/43 mM M is very hypertonic, and infusion of high volumes of 4 M BHB/43 mM M markedly increases the number of ions that can exit the vasculature, increasing interstitial volume load. There were numerous clinical indicators of third spacing in the studied pigs, including hypovolemia, increased intraabdominal pressure and organ dysfunction.

Third-spacing results in hypovolemia, hypotension and decreased cardiac output (325). In our study, infusion of above standard doses of 4 M BHB/43 mM M resulted in dose-dependent decreases in MAP (Figure 3.2). Falsely elevated hemoglobin levels due to hemoconcentration in 4x 4 M BHB/43 mM M pigs before death further indicate

hypovolemia (Table 3.2, (325)). The decrease in intravascular volume was observed despite increased administration of resuscitation fluids, as indicated by a higher net fluid balance (Figure 3.2).

Interstitial fluid accumulation leads to increased intraabdominal pressure (IAP) and edema in various tissues (326-328). IAP can be determined by measuring bladder pressure, and a bladder pressure above 12 mm Hg is commonly considered intraabdominal hypertension (329). In our study, all groups experienced intraabdominal hypertension at some point during resuscitation, however, IAP was highest in animals receiving increased 4 M BHB/43 mM M doses, where it greatly exceeded physiological levels (30 mm Hg in 4x 4 M BHB/43 mM M pigs, Figure 3.6). Increases in mean pulmonary artery pressure and wedge pressure further indicate increased IAP in this group (Figure 3.2, table 3.2, (330, 331)).

Elevated IAP decreases abdominal perfusion pressure and impairs tissue perfusion (332). High IAP is associated with organ dysfunction, including in the kidney, the lungs and the liver, which becomes more grave with increasing pressure (329-331, 333). In our study, pigs treated with 2x 4 M BHB/43 mM and 4x 4 M BHB/43 mM M experienced dose-dependent increases in lactate and lactate dehydrogenase (LDH), markers of tissue ischemia and injury (334). Ketone body infusion is associated with increased serum pH in both healthy volunteers and trauma patients (335, 336). However, lactic acidosis resulted in decreased pH in pigs receiving 4x 4M BHB/ 43mM M (Figure 3.5). Decreases in PaO_2:FiO_2 ratios in pigs infused with 2x 4 M BHB/43 mM or 4x 4 M BHB/43 mM M indicate impaired blood oxidation and pulmonary dysfunction (333). Raised AST levels indicate increased liver injury. Organ dysfunction may have been exacerbated by hypernatremia, which itself has been associated with pulmonary and peripheral edema (337-339).

In our study, elevated CK levels indicate ischemia-induced muscle injury and rhabdomyolysis, which can cause acute renal failure (340). Furthermore, third spacing can lead to acute kidney injury due to intravascular volume depletion-induced acute tubular necrosis, the release of cytokines or increased IAP (326, 341-343). Urine output was decreased in 4x 4 M BHB/43 mM M animals, indicating renal dysfunction despite the highest rate of fluid administration in this group. Decreased urine output impairs the resolution of intraabdominal hypertension and further exacerbates hypernatremia by preventing sodium excretion (328, 344).

Translocation of intravascular fluid to the interstitial space leads to hypovolemia and hypotension (325). To mimic the clinical setting, our protocol used blood pressure, urine output and hemoglobin as resuscitation endpoints to guide fluid administration (Figure 3.1, (31, 345)). When these endpoints are not met due to intravascular fluid loss, increased fluid administration leads to fluid overload, which further increase IAP and has been associated with adverse outcomes (328, 346, 347). Indeed, lowered MAP in pigs receiving 2x 4 M BHB/43 mM M and 4x 4 M BHB/43 mM M resulted in increased fluid administration during resuscitation in these groups, as indicated by the cumulative net fluid balance (Figure 3.2).

Combined, our results suggest that above-standard doses of 4 M BHB/43 mM M induced significant hypernatremia and intravascular fluid loss to the interstitial space. Death was likely a result of the interplay of hemorrhagic shock, injury, hypernatremia, intravascular fluid loss and organ failure. While these effects were reversed in some of the 2x 4 M BHB/43 mM M treated pigs, they were irreversible when 4x 4 M BHB/43 mM M was given. We conclude that in our porcine model of hemorrhagic shock, injury and resuscitation, 2x 4 M BHB/43 mM M is the maximum tolerated dose. In our study, although we saw increased mortality in 2x 4 M BHB/43 mM M animals, hypernatremia and third-spacing resolved in surviving animals in this group. As the MTD, 2x 4 M BHB/43 mM M will likely reveal any long term adverse effects of increased sodium levels and intraabdominal hypertension in preclinical toxicity studies.

Objective 2: D-β-Hydroxybutyrate and Melatonin for Treatment of Porcine Hemorrhagic Shock and Injury: A Melatonin Dose-Ranging Study

Results

Shock induction and resuscitation

There were no significant differences in the amount of blood withdrawn, blood returned or total fluids administered among the treatment groups. Pigs treated with 4 M BHB/10 mM M received significantly less LR than those receiving 4 M BHB/0.43 mM M (95% CIs [750,2206], [2344, 4908] ml/kg, $p=0.029$), which is likely due to the high early mortality in this group (Figure 3.7).

Survival

Twenty-four hours after extubation there was a significant difference in overall survival ($p=0.017$, Figure 3.7), with the highest rate observed in the 4 M BHB/43 mM M group (13/14), followed by LR pigs (10/16) and those receiving lower doses of melatonin (4 M BHB/20mM M 3/6, 4 M BHB/10 mM M 2/6, 4 M BHB/4.3 mM M 3/6, 4 M BHB/0.43 mM M 1/6). Survival between 4 M BHB/43 mM M and LR-treated pigs did not differ significantly ($p = 0.094$). There were significant differences between the 43 mM and the 20 mM, 10mM and 0.43 mM M groups ($p<0.05$), and between the LR and the 4 M BHB/10 mM M group ($p = 0.028$).

Drug serum levels

BHB/M-treated pigs experienced dose-dependent increases in melatonin and BHB serum concentrations, which peaked at the end of shock and returned to control levels by the end of resuscitation (Figure 3.8). Melatonin concentrations in 4 M BHB/43 mM M pigs were significantly higher than in all other groups after shock and during early resuscitation. Differences were significant between pigs infused with 4 M BHB/20 mM M versus those receiving LR, 4 M BHB/4.3 mM M or 4 M BHB/0.43 mM M at the end of shock. At the end of shock, BHB concentrations were higher in all BHB/M groups than in controls. We observed some variability in BHB levels which resulted in significant group differences, however, there was no obvious effect of melatonin dose on

BHB serum concentrations. Total drug exposure over time followed the patterns observed for drug serum levels.

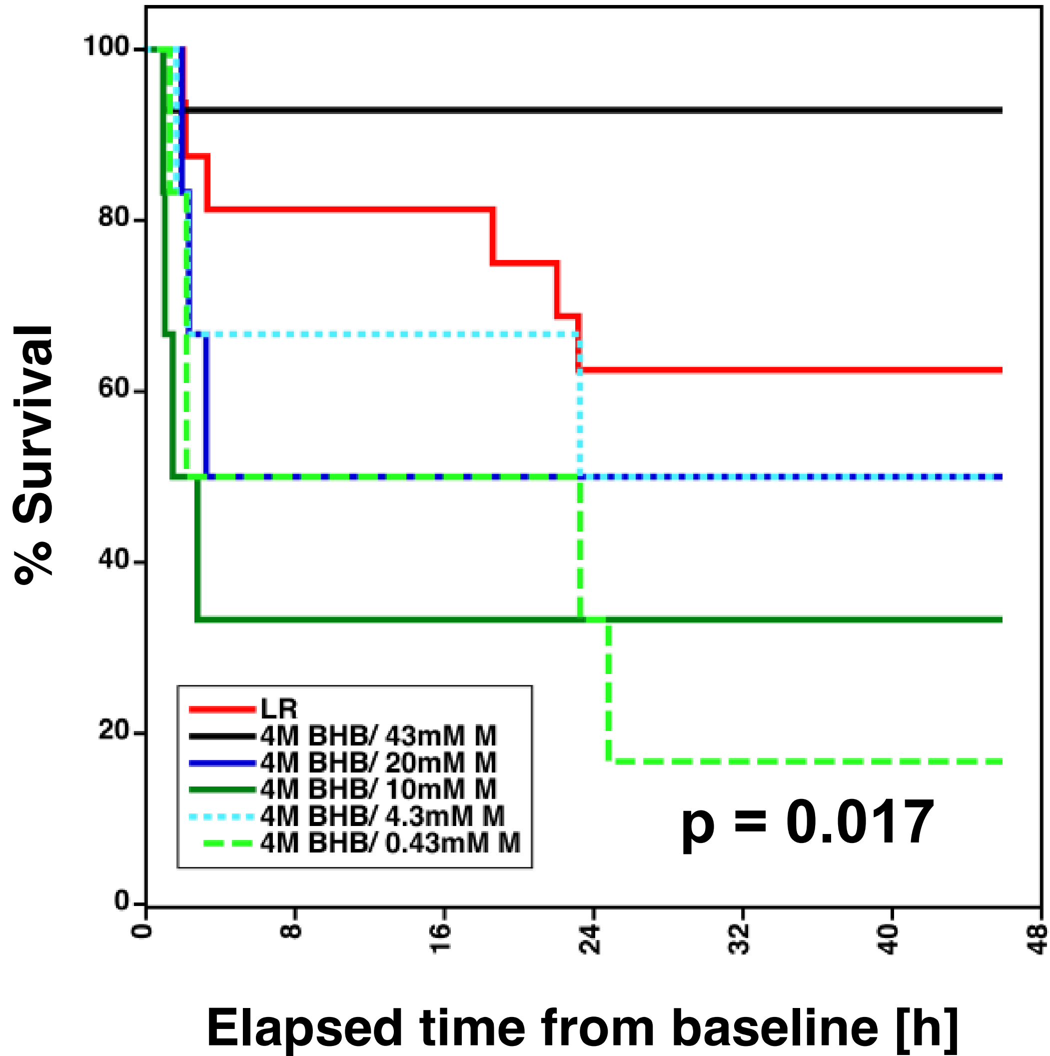


Figure 3.7. Survival in pigs experiencing hemorrhagic shock and injury. Mean survival in hours [95% CI]: LR 33.1 [24.5, 41.6], 4 M BHB/43 mM M 42.6 [36.5, 48.6], 4 M BHB/20 mM M 24.1 [6.8, 41.4], 4 M BHB/10 mM M 16.3 [0, 33.0], 4 M BHB/4.3 mM M 27.4 [11.7, 43.2], 4 M BHB/0.43 mM M 16.6 [3.5, 29.7].

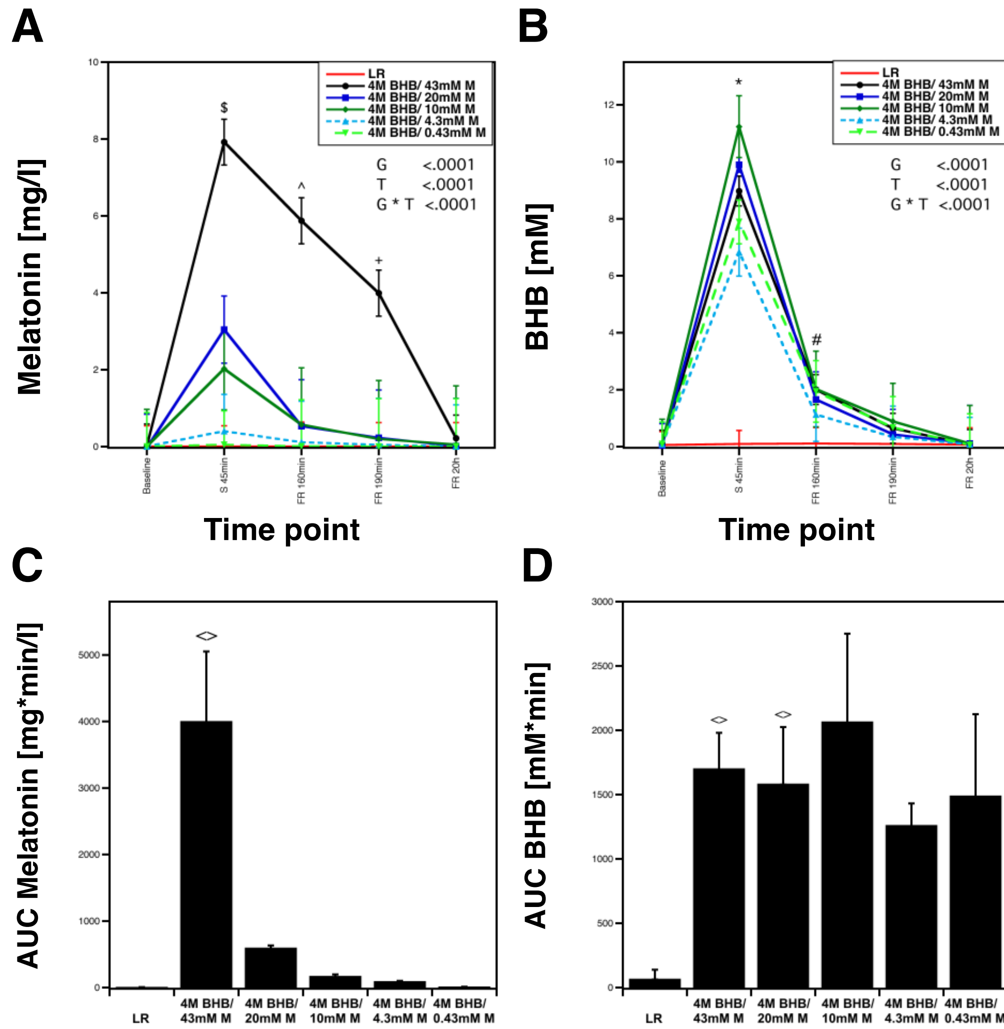


Figure 3.8. (A, B) Average drug serum levels and (C, D) drug exposure over time during hemorrhagic shock and injury. Data presented as least-squares means with 95% confidence intervals (A, B) or as medians with IQR (C, D). AUC Melatonin in mg*min/l [95% CI]: LR [7, 7], 4 M BHB/43 mM M [1418, 6852], 4 M BHB/20 mM M [411, 633], 4 M BHB/10 mM M [145, 198], 4 M BHB/4.3 mM M [73, 108], 4 M BHB/0.43 mM M [11, 13]. AUCt BHB in mM*min [95% CI]: LR [25, 184], 4 M BHB/43 mM M [1026, 3586], 4 M BHB/20 mM M [1578, 2025], 4 M BHB/10 mM M [1383, 2751], 4 M BHB/4.3 mM M [888, 1454], 4 M BHB/0.43 mM M [1169, 2126]. $ p<0.05 for 4 M BHB/43 mM M vs all other treatments and 4 M BHB/20 mM M vs LR, 4 M BHB/4.3 mM M and 4 M BHB/0.43 mM M; ^ p<0.05 for 4 M BHB/43 mM M vs all other treatments; + p<0.05 for 4 M BHB/43 mM M vs all other treatments; * p<0.0.5 for LR vs all other treatments, 4 M BHB/4.3 mM M vs 4 M BHB/43 mM M and 4M BHB/20 mM M and 4 M BHB/10 mM M, 4M BHB/10 mM M vs 4 M BHB/0.43 mM M; # p<0.05 for LR vs 4 M BHB/43 mM M; <> p<0.05 vs LR.

Hemodynamics, serum electrolytes, blood gases and markers of organ function

Key hemodynamic and physiologic parameters are depicted in Table 3.3. Hemorrhage caused a drop in MAP and cardiac output along with increases in heart rate in all groups, which recovered during resuscitation. Urine output did not differ significantly between groups at individual time points. BHB/M treatment increased sodium and decreased potassium levels during early resuscitation, a previously described effect that was independent of treatment melatonin concentration (232). We observed shock-induced decreases in pH which returned towards baseline levels during resuscitation. There were no obvious BHB/M-treatment or melatonin dose-dependent effects on hemoglobin or serum levels of blood urea nitrogen and LDH. There were no obvious treatment-dependent effects on body temperature, mean pulmonary artery pressure, pulmonary artery occlusion pressure, bladder pressure, mixed venous oxygen, oxygen consumption, serum levels of alanine aminotransferase, albumin, total protein, bilirubin and alkaline phosphatase (not shown).

Lactate levels peaked during limited resuscitation but returned to baseline levels by the end of the experiment (Figure 3.9 A). BHB/M-treated pigs receiving low melatonin concentrations experienced dose-dependent decreases in PaO_2:FiO_2 ratios during early resuscitation (Figure 3.9 B). Pigs treated with BHB/M experienced increases in serum concentrations of AST and CK (Figure 3.9 C, D). The shock-induced disturbances were most prominent in groups with high early mortality rates, namely in pigs receiving 4 M BHB with 20 mM, 10 mM and 0.43 mM melatonin.

8-isoprostane urine levels were analyzed as markers of trauma-induced oxidative stress (348). There was an insignificant trend towards lower 8-isoprostane levels in the groups treated with BHB/M during resuscitation (Figure 3.10). This effect was independent of the melatonin concentration in the treatment.

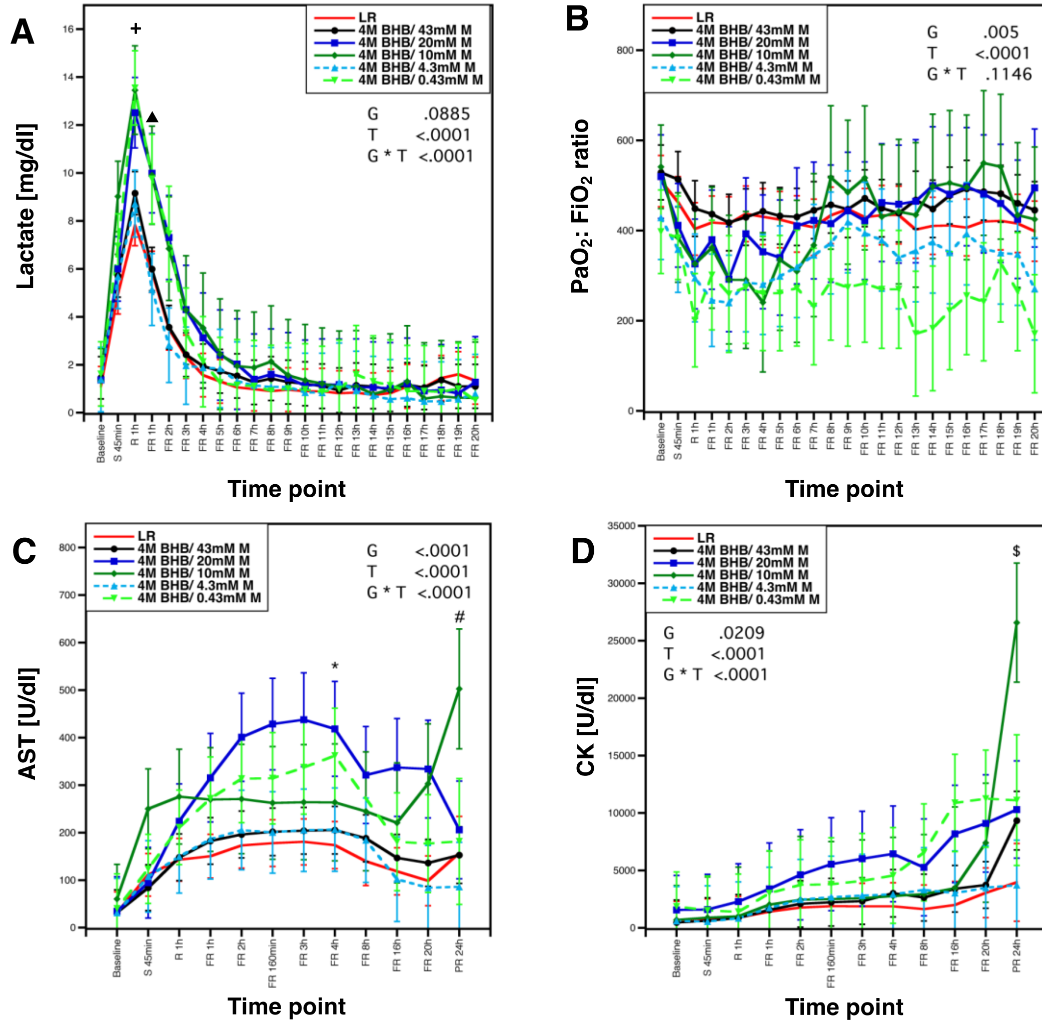


Figure 3.9. (A) Lactate levels, (B) PaO_2:FiO_2 ratios, (C) AST levels and (D) CK concentrations throughout the experiment. Data presented as least-squares means with 95% CIs. + $p<0.05$ for LR vs 4 M BHB/10 mM M; ▲ $p<0.05$ for LR vs 4 M BHB/20 mM M and 4 M BHB/10 mM M and 4 M BHB/0.43 mM M, 4 M BHB/0.43 mM M vs 4 M BHB/43 mM M and 4 M BHB/4.3 mM M. * $p<0.05$ for LR vs 4 M BHB/20 mM M; # for 4 M BHB/10 mM M versus 4 M BHB/4.3 mM M and 4 M BHB/43 mM M; $ for 4 M BHB/10 mM M vs LR and 4 M BHB/43 mM M and 4 M BHB/20 mM M and 4 M BHB/4.3 mM M.

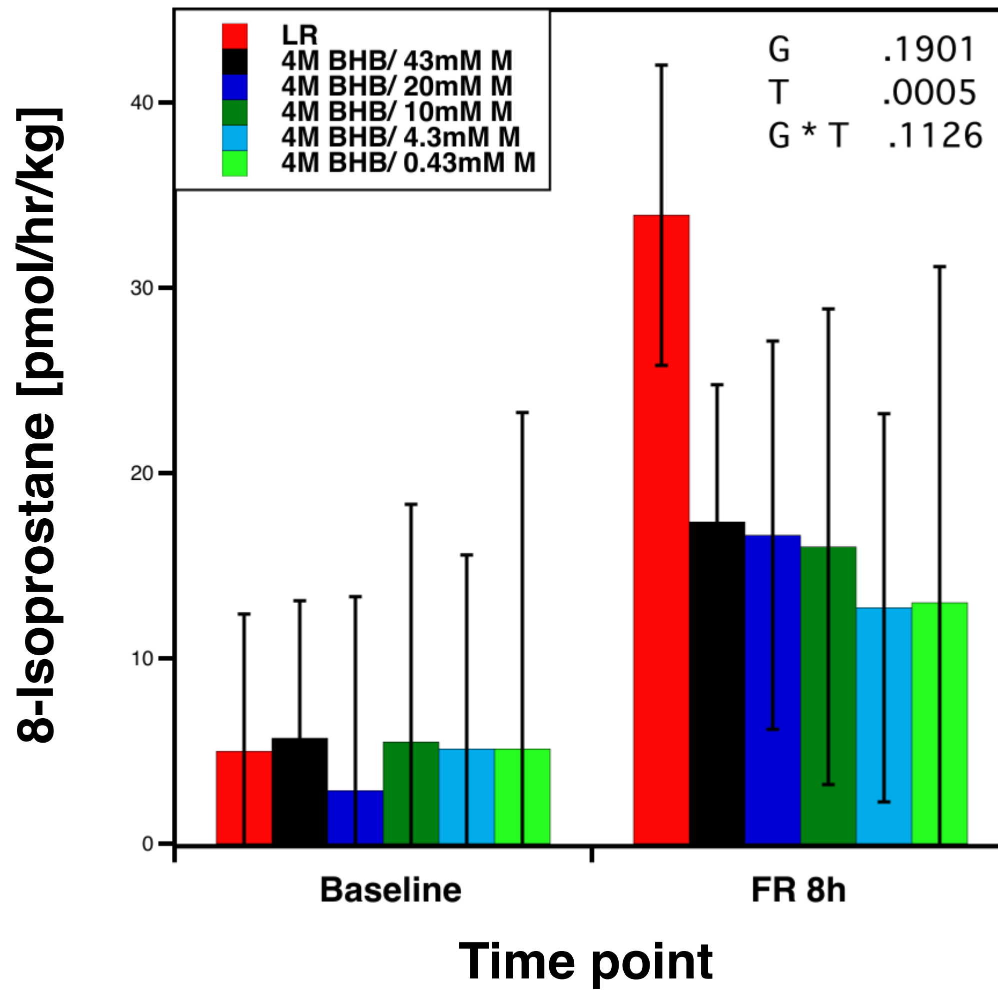


Figure 3.10. Urine 8-Isoprostane levels during hemorrhagic shock and injury. Data are presented as least-squares means with 95% CIs.

Table 3.3. Physiologic parameters and markers of organ function in pigs exposed to hemorrhagic shock, injury and resuscitation.

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 24 h
MAP [mm Hg]			Effects: G 0.0071 T <.0001 G*T 0.0091			
LR	88 (82-94)	41 (35-47)	58 (52-64)	74 (67-80)	68 (61-76)	
4 M BHB/43 mM M	85 (78-91)	41 (34-47)	59 (52-65)	73 (66-80)	72 (65-79)	
4 M BHB/20 mM M	80 (70-90)	40 (31-50)	44 (33-55)	66 (52-79)	67 (53-81)	
4 M BHB/10 mM M	92 (82-102)	35 (23-46)	62 (47-76)	64 (48-81)	96 (79-113)	
4 M BHB/4.3 mM M	88 (78-98)	34 (24-43)	48 (38-58)	65 (53-76)	66 (54-78)	
4 M BHB/0.43 mM M	95 (85-105)	44 (34-54)	48 (37-59)	67 (52-82)	59 (45-73)	
Heart rate [beats/ min]			Effects: G 0.0002 T <.0001 G*T 0.0015			
LR	120 (104-136)	237 (222-253)	175 (159-192)	169 (152-186)	158 (139-177)	
4 M BHB/43 mM M	125 (108-142)	198 (181-215)	179 (161-196)	151 (133-168)	144 (126-161)	
4 M BHB/20 mM M	137 (111-163)	254 (228-279)	205 (176-234)	217 (182-253)	146 (110-183)	
4 M BHB/10 mM M	135 (109-161)	250 (220-279)	197 (158-237)	186 (142-230)	153 (108-198)	
4 M BHB/4.3 mM M	144 (118-170)	218 (193-244)	147 (120-174)	153 (122-184)	126 (94-158)	
4 M BHB/0.43 mM M	151 (125-177)	264 (239-290)	189 (160-219)	198 (158-239)	166 (130-202)	

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 24 h
Cardiac Output [l/min]			Effects: G 0.0157 T <.0001 G*T 0.1389			
LR	2.9 (2.4-3.4)	1.3 (0.9-1.8)	2.9 (2.4-3.5)	4.4 (3.9-5.0)	4.3 (3.7-4.9)	
4 M BHB/43 mM M	2.9 (2.4-3.4)	1.7 (1.1-2.3)	4.1 (3.6-4.7)	5.1 (4.5-5.6)	4.0 (3.5-4.6)	
4 M BHB/20 mM M	3.0 (2.3-3.8)	1.5 (0.7-2.3)	2.3 (1.3-3.3)	4.2 (3.1-5.3)	3.2 (2.1-4.3)	
4 M BHB/10 mM M	3.2 (2.4-3.9)	0.9 (0.1-1.8)	2.3 (1.1-3.5)	3.6 (2.3-5.0)	3.8 (2.5-5.1)	
4 M BHB/4.3 mM M	3.3 (2.5-4.1)	1.4 (0.6-2.1)	3.6 (2.7-4.5)	4.6 (6.7-5.6)	3.4 (2.4-4.3)	
4 M BHB/0.43 mM M	4.0 (3.2-4.7)	1.2 (0.4-1.9)	2.5 (1.5-3.5)	5.3 (4.0-6.6)	4.5 (3.2-5.7)	
Urine Output [ml/h]			Effects: G 0.0625 T <.0001 G*T 0.0911			
LR	39 (-10-88)	18 (-31-66)	71 (20-122)	87 (35-140)	107 (47-168)	
4 M BHB/43 mM M	63 (11-114)	17 (-36-69)	132 (79-185)	152 (99-206)	140 (87-194)	
4 M BHB/20 mM M	20 (-58-99)	18 (-61-97)	37 (-52-127)	72 (-36-180)	41 (-71-152)	
4 M BHB/10 mM M	92 (14-171)	5 (84-95)	45 (-74-164)	75 (-58-209)	89 (-47-226)	
4 M BHB/4.3 mM M	51 (-28-129)	17 (-62-95)	155 (72-238)	75 (-19-170)	120 (24-216)	
4 M BHB/0.43 mM M	67 (-12-145)	22 (-56-101)	38 (-51-128)	200 (73-327)	84 (-27-195)	
Na^+ [mEq/l]			Effects: G <.0001 T <.0001 G*T 0.4134			
LR	139.0 (136.7-141.4)	138.4 (136.0-140.7)	139.5 (136.9-142.0)	137.7 (135.1-140.3)	142.1 (139.2-145.1)	
4 M BHB/43 mM M	139.2 (136.7-141.7)	143.8 (141.2-146.4)	145.6 (143.0-148.2)	148.2 (146.5-150.0)	142.9 (140.3-145.5)	
4 M BHB/20 mM M	140.5 (136.7-144.3)	145.2 (141.3-149.0)	147.7 (143.2-152.3)	150.2 (144.8-155.6)	144.0 (138.6-149.4)	
4 M BHB/10 mM M	140.5 (136.7-144.3)	144.3 (139.7-148.8)	150.5 (144.2-156.9)	149.3 (142.7-156.0)	152.5 (145.9-159.1)	
4 M BHB/4.3 mM M	142.8 (139.0-146.7)	144.3 (140.5-148.2)	146.8 (142.7-151.0)	148.9 (144.2-153.6)	146.0 (141.3-150.7)	
4 M BHB/0.43 mM M	140.7 (136.8-144.5)	145.0 (141.2-148.8)	148.1 (143.0-153.2)	149.8 (143.3-156.4)	144.3 (138.9-149.8)	

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 24 h
K^+ [mEq/l]			Effects: G <.0001 T <.0001 G*T <.0001			
LR	3.9 (3.7-4.2)	5.8 (5.6-6.1) [#]	3.9 (3.7-4.2) [#]	4.3 (4.1-4.6) [#]	3.3 (3.0-3.6)	
4 M BHB/43 mM M	4.0 (3.7-4.2)	4.6 (4.3-4.8) [*]	3.0 (2.7-3.3) [*△]	3.2 (2.9-3.4) [*○]	3.3 (3.0-3.5)	
4 M BHB/20 mM M	3.8 (3.4-4.2)	5.0 (4.6-5.4)	4.4 (4.0-4.9) [#]	3.6 (3.0-4.1)	3.0 (2.5-3.6)	
4 M BHB/10 mM M	3.8 (3.4-4.2)	5.5 (5.0-6.0)	3.1 (2.4-3.7)	3.2 (2.6-3.9)	2.6 (1.9-3.3)	
4 M BHB/4.3 mM M	3.8 (3.4-4.2)	5.0 (4.6-5.4)	3.0 (2.6-3.4)	3.0 (2.6-3.5) [*]	3.5 (3.0-3.9)	
4 M BHB/0.43 mM M	4.0 (3.6-4.4)	5.7 (5.3-6.0)	3.5 (3.0-4.1)	3.0 (2.4-3.7)	3.0 (2.5-3.6)	
pH			Effects: G <.0001 T <.0001 G*T <.0001			
LR	7.47 (7.43-7.50)	7.39 (7.35-7.42)	7.28 (7.25-7.32) [○●]	7.38 (7.34-7.41) [#]	7.42 (7.38-7.46)	
4 M BHB/43 mM M	7.45 (7.42-7.49)	7.44 (7.40-7.47)	7.38 (7.34-7.41) [●]	7.50 (7.47-7.54) [*]	7.48 (7.44-7.51)	
4 M BHB/20 mM M	7.46 (7.40-7.51)	7.39 (7.33-7.45)	7.25 (7.18-7.31)	7.36 (7.29-7.44)	7.49 (7.41-7.57)	
4 M BHB/10 mM M	7.47 (7.41-7.53)	7.39 (7.33-7.45)	7.28 (7.20-7.36)	7.43 (7.34-7.53)	7.45 (7.36-7.55)	
4 M BHB/4.3 mM M	7.48 (7.42-7.53)	7.44 (7.39-7.50)	7.44 (7.38-7.50) [*●]	7.51 (7.45-7.58)	7.39 (7.32-7.46)	
4 M BHB/0.43 mM M	7.45 (7.39-7.51)	7.43 (7.37-7.48)	7.07 (7.01-7.14) [*#○]	7.29 (7.20-7.37)	7.36 (7.28-7.43)	
Hemoglobin [g/dl]			Effects: G 0.014 T <.0001 G*T 0.1014			
LR	8.7 (8.4-9.0)	7.0 (6.6-7.3)	5.2 (4.8-5.5)	6.1 (5.7-6.4)	5.7 (5.3-6.1)	
4 M BHB/43 mM M	8.6 (8.2-8.9)	6.1 (5.8-6.4)	5.0 (4.6-5.3)	6.0 (5.7-6.4)	5.6 (5.3-6.0)	
4 M BHB/20 mM M	8.6 (8.0-9.1)	6.3 (5.8-6.8)	5.6 (5.0-6.2)	6.4 (5.7-7.1)	6.1 (5.4-6.8)	
4 M BHB/10 mM M	8.7 (8.1-9.2)	5.9 (5.3-6.5)	5.3 (4.5-6.1)	6.5 (5.6-7.4)	6.7 (5.8-7.5)	
4 M BHB/4.3 mM M	8.9 (8.3-9.4)	6.5 (5.9-7.0)	5.2 (4.5-5.9)	6.1 (5.4-6.7)	6.0 (5.3-6.6)	
4 M BHB/0.43 mM M	8.6 (8.0-9.1)	6.9 (6.4-7.4)	4.5 (3.7-5.2)	5.9 (5.1-6.7)	5.7 (5.0-6.4)	
4 M BHB/0.43 mM M	9.5 (-11.8-30.8)	11.7 (9.6-32.9)	8.5 (-17.5-34.5)	10.7 (-19.4-40.7)	11.3 (-18.7-41.4)	

Parameter	Baseline	S 45 min	R 1 h	FR 2 h	FR 20 h	PR 24 h
Blood urea nitrogen [mg/dl]			Effects: G 0.0095 T 0.0992 G*T <.0001			
LR	13.6 (0.6-26.6)	11.3 (-2.1-24.8)	10.6 (-2.8-24.0)	12.8 (-2.2-27.9)	31.5 (15.8-47.2)	16.0 (-20.8-52.8) △
4 M BHB/43 mM M	9.5 (-4.4-23.4)	11.9 (-2.5-26.4)	10.8 (-4.3-25.8)	12.6 (-1.8-27.1)	14.4 (-0.1-28.8)	13.0 (-13.0-39) △
4 M BHB/20 mM M	8.8 (-12.4-30.1)	11.2 (-12.1-34.5)	12.3 (-13.8-38.3)	15.0 (-15.1-45.1)	17.7 (-12.4-47.7)	207.0 (176.9-237.1) *#▲○●
4 M BHB/10 mM M	9.8 (-11.4-31.1)	11.8 (-14.3-37.8)	8.0 (-28.8-44.8)	9.0 (-27.8-45.8)	12.5 (-24.3-49.3)	8.0 (-28.8-44.8) △
4 M BHB/4.3 mM M	8.3 (-12.9-29.6)	11.6 (-11.7-34.9)	9.6 (-13.7-32.9)	11.8 (-14.3-37.8)	11.8 (-14.3-37.8)	16.0 (-14.1-46.1) △
4 M BHB/0.43 mM M	9.5 (-11.8-30.8)	11.7 (9.6-32.9)	8.5 (-17.5-34.5)	10.7 (-19.4-40.7)	11.3 (-18.7-41.4)	11.0 (-41.1-63.1) △
LDH [U/dl]			Effects: G 0.0005 T <.0001 G*T <.0001			
LR	1122 (518-1725)	1320 (715-1924)	1406 (801-2011)	1882 (1267-2497)	2801 (2132-3469) ●	3279 (2524-4035) ●
4 M BHB/43 mM M	1098 (453-1744)	1168 (521-1816)	1445 (795-2095)	2431 (1779-3083)	3065 (2399-3731) ●	4563 (3876-5259) △
4 M BHB/20 mM M	560 (-426-1546)	589 (-401-1580)	908 (-97-1913)	1296 (226-2366)	1480 (153-2807) ●	1307 (-25-2639) #●
4 M BHB/10 mM M	501 (-485-1487)	638 (-384-1660)	854 (-234-1943)	877 (-366-2120)	1457 (-148-3062) ●	1294 (-385-2973) ●
4 M BHB/4.3 mM M	1266 (280-2252)	1630 (637-2623)	1659 (659-2659)	2630 (1597-3662)	2863 (1693-4033) ●	3579 (2391-4767) ●
4 M BHB/0.43 mM M	1452 (465-2438)	1551 (565-2537)	1983 (978-2987)	3170 (2101-4239)	7857 (6530-9184) *#△▲○	7681 (6244-9118) *△▲○

Data are presented as least-squared means (95% confidence interval). * $p<0.05$ vs LR, # $p<0.05$ vs 4M BHB/43mM M, △ $p<0.05$ vs 4M BHB/20mM M, ▲ $p<0.05$ vs 4M BHB/10mM M, ○ $p<0.05$ vs 4M BHB/4.3mM M, ● $p<0.05$ vs 4M BHB/0.43mM M.

Discussion

4 M BHB/43 mM M significantly improves survival in preclinical hemorrhagic shock models (231, 232). Here, we describe experiments to optimize the melatonin concentration in the treatment. In rats, lowering the BHB concentration in combination with 43 mM M resulted in a trend towards decreased survival, while survival times were retained when melatonin levels were lowered (233). Based on these results, we used our porcine hemorrhage, injury and resuscitation model to evaluate treatment solutions containing 4 M BHB in combination with 0.43-43 mM melatonin. We hypothesized that the melatonin concentration could be decreased without loss of efficacy.

Mortality in pigs receiving BHB/M containing below-standard melatonin concentrations exceeded that in the control group (Figure 3.7). This was surprising, as previous studies showed that a decrease in melatonin concentration in combination with BHB was inconsequential in rat hemorrhagic shock (233). In rats experiencing hemorrhagic shock, 4 M BHB alone significantly improved survival, and lowering the BHB concentration in BHB/M was associated with a trend towards increased mortality (231, 233). Together, these studies suggest that BHB exerts beneficial effects in hemorrhagic shock, acting synergistically with melatonin.

Our data indicates that treatment with BHB/M containing decreased melatonin levels increased shock-induced lung and organ injury. These animals experienced increased lactate levels and elevated serum concentrations of AST and CK, markers of liver and muscle injury (Figure 3.9). Pulmonary contusion and hemorrhagic shock induce pulmonary inflammation, which can lead to hypoxemia and acute respiratory distress syndrome (349, 350). We observed melatonin dose-dependent decreases in PaO_2:FiO_2 ratios during early resuscitation, indicating increased lung injury. This was unexpected, as both melatonin and BHB exhibit anti-inflammatory effects and BHB decreases inflammation and apoptosis in rats and pigs exposed to severe blood loss (154, 189, 351-358). However, melatonin effects in hemorrhagic shock can be dose-dependent, and systemic or local melatonin concentrations may need to overcome a threshold level to exert beneficial effects. For example, infusion of 50 mg/kg, but not of 10 mg/kg melatonin prior to resuscitation resulted in lowered resuscitation fluid requirements and decreased serum levels of the inflammatory marker IL-6 in mouse hemorrhagic shock (188). Furthermore, ketone bodies may be pro-inflammatory at high doses (188, 359-361).

With systemic melatonin levels insufficient to counteract the effects of shock and injury, the high BHB dose may have exacerbated trauma-induced inflammation.

BHB metabolism generates NADH, which acts as a reducing equivalent for the mitochondrial electron transport chain and ATP production. A significant increase in the NADH/NAD^+ ratio during limited oxygen supply can overwhelm the electron transport chain, thereby inducing production of reactive oxygen species (362). To test whether increased mortality in the low-dose melatonin group was a result of decreased counteraction of BHB-induced oxidative stress, we measured urine levels of 8-isoprostane (348). We observed an insignificant trend towards decreased 8-isoprostane levels in BHB/M-treated pigs, an effect that was independent of the melatonin concentration in the treatment (Figure 3.10). This suggests that rather than inducing oxidative damage, BHB decreased oxidative stress in our model.

Limitations

Our experiments have some limitations. As is common for MTD dose-finding studies, we used a small number of animals in each group, which may have masked some significant effects. We did not evaluate inflammatory markers in these animals, and while inflammation is a likely contributor to the pathology observed in this study, the mechanisms behind third spacing in our model remain speculative.

As the experiments were an extension of a previous study, we expanded the original 4 M BHB/43 mM M and LR groups and added treatments to our original experiment (293). Consequently, group sizes were uneven and animals were not completely randomized, rendering a risk for model variation over time. However, 4 M BHB/43 mM M (the standard dose) exerted a robust beneficial effect and consistently outperformed LR in our model (2/2 additional BHB/M pigs survived, while only 1/4 of LR pigs survived). Survival differences between 4 M BHB/43 mM M and the control group were not significant, which is likely due to the limited sample size used. As our experiments clearly showed that decreasing melatonin concentrations was detrimental, we limited our sample size to save animals and resources

As we did not include DMSO-treated control groups in our experiments, it is unclear whether changes in DMSO concentrations affected efficacy in the low-dose melatonin groups. Previously, BHB/M treatment was significantly more effective at increasing post-shock survival than treatment with isosmotic solutions containing equal DMSO concentrations (232). As we concluded that it was unlikely that changes in DMSO concentration affected survival, we opted for LR as control to represent the standard of care.

Summary and Conclusions

The experiments presented in this chapter suggest that, in the current formulation, BHB/M has a relatively small therapeutic window in our model of porcine hemorrhagic shock, injury and resuscitation. However, our study also indicates that the toxicity of 2x 4 M BHB/43 mM M and 4x 4 M BHB/43 mM M is likely not due to BHB or melatonin per se, but a result of the infusion of large volumes of a hypertonic solution with a high sodium content. Consequently, treatment toxicity may be decreased by lowering solution tonicity or limiting the amount of sodium in the formulation by utilization of alternative BHB salts. An alternative means to induce ketosis in hemorrhagic shock is the administration of ketone body precursors. Different ketone esters have been developed, which release BHB or acetoacetate after hydrolysis in the liver or intestine (363). Ketone ester infusion in rats, pigs or dogs increased systematic ketone levels without adverse effects (364-367). Clarke et al. showed that ingestion of the ketone ester R-3-hydroxybutyl R-3-hydroxybutyrate was generally well tolerated in human volunteers (368). Ketone esters are sodium free, therefore, ketone ester infusion would not produce the sodium load induced by infusion of Na-D-β-hydroxybutyrate. Furthermore, as one ketone ester molecule releases two or three molecules of ketones (366-368), ketone ester solutions would be less hypertonic than an equal BHB dose infused as Na-D-β-hydroxybutyrate. Ketone esters therefore have the potential to avoid the hypernatremia and hypertonicity-associated adverse effects of BHB/M in the current formulation. Switching to non-sodium fluids during the resuscitation phase (e.g. albumin, dextrose solution) would also decrease sodium load. Optimization of resuscitation endpoints and application of a more restrictive resuscitation protocol after BHB/M infusion may limit fluid overload and improve outcomes (328). Targeted use of diuretics or renal replacement therapy after initial resuscitation may decrease sodium toxicity without loss of the initial beneficial effects of BHB/M (328).

We also tested the efficacy of 4 M BHB in combination with 0.43-43 mM melatonin in porcine hemorrhagic shock, injury and resuscitation. Treatment with below-standard melatonin concentrations resulted in mortality rates exceeding that in the control group. Lowered melatonin treatment concentrations resulted in increased markers of lung, liver and kidney injury, suggesting that decreased melatonin serum levels were insufficient to counteract BHB-induced increases in inflammation. Our research underlines the importance of reaching adequate systemic BHB and melatonin levels. Consequently,

lowering the amount of DMSO in the formulation by decreasing the melatonin concentration in the treatment is not feasible without loss of efficacy.

Chapter 4. Evaluation of Novel Formulations of D-β-hydroxybutyrate and Melatonin in a Rat Model of Hemorrhagic Shock

This chapter has been submitted for publication:

Wolf A, Thakral S, Mulier KE, Suryanarayanan R, Beilman GJ. Evaluation of Novel Formulations of D-β-hydroxybutyrate and Melatonin in a Rat Model of Hemorrhagic Shock.

Synopsis

Infusion of D-β-hydroxybutyrate and melatonin (BHB/MLT) improves survival in hemorrhagic shock models. The original BHB/MLT formulation contains dimethyl sulfoxide (DMSO) to overcome the limited aqueous solubility of melatonin. We formulated two BHB/MLT solutions wherein DMSO was replaced either with 10% polyvinylpyrrolidone K12 (BHB/MLT/PVP) or with 5% hydroxypropyl-β-cyclodextrin/2.5% PVP/2.5% polyethylene glycol 400 (BHB/MLT/CD). The safety and efficacy of the new BHB/MLT formulations and the original solution (full strength and 1:1 dilution) were tested in a lethal rat hemorrhagic shock model, consisting of seven groups: 1) sham, 2) shock, untreated, 3) shock, lactated Ringer's solution (LR), 4) shock, 4 M BHB/MLT/DMSO, 5) shock, 2 M BHB/MLT/DMSO, 6) shock, BHB/MLT/PVP and 7) shock, BHB/MLT/CD. Rats were anesthetized and instrumented, after which 40% of the total blood volume was withdrawn in three steps. Rats were in hemorrhagic shock for four hours. Treatments were administered as a bolus infusion half-way throughout hemorrhage. In hemorrhaged animals, survival was highest in the BHB/MLT/CD group (8/10), followed by treatment with BHB/MLT/PVP (6/10), 4M BHB/MLT/DMSO (5/10) or 2M BHB/MLT/DMSO (5/10), LR (3/10) and the untreated group (0/11). Survival did not differ significantly between the four BHB/MLT treatment groups ($p>0.05$). Survival in BHB/MLT/CD-treated rats was significantly higher than in the LR group ($p = 0.018$). BHB/MLT/PVP and BHB/MLT/CD caused significantly less intravascular hemolysis than 4 M BHB/MLT/DMSO. Markers of toxicity did not differ significantly between the four BHB/MLT formulations. Our experiments indicate that BHB/MLT/PVP and BHB/MLT/CD constitute promising candidates for the clinical treatment of acute hemorrhagic shock.

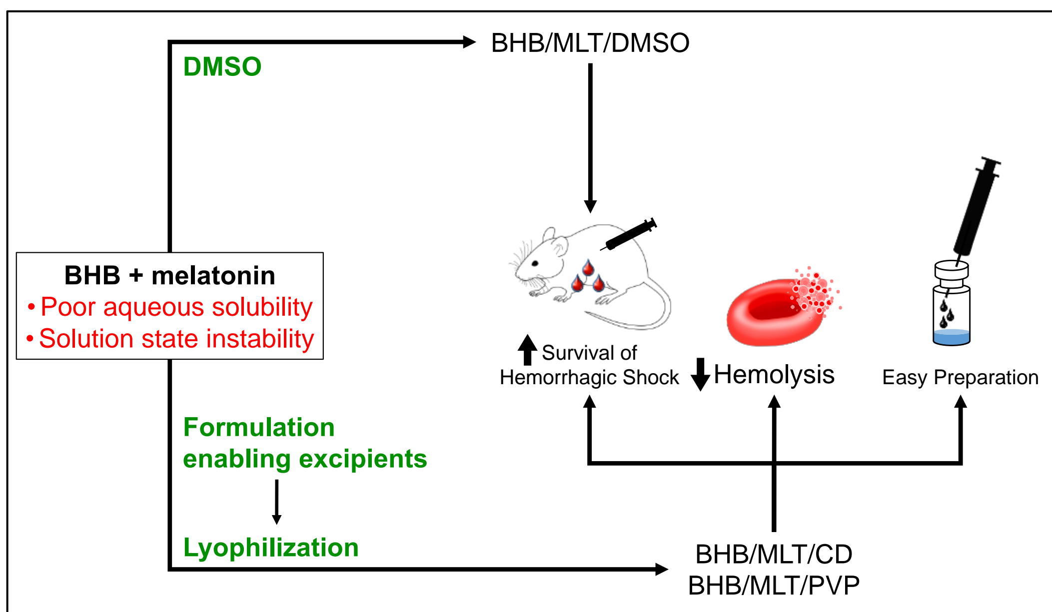


Figure 4.1. Graphical Synopsis.

Introduction

It is estimated that one third to more than 50% of deaths after hemorrhagic shock occur during the pre-hospital phase (3). Many of these deaths may be preventable with adequate treatment during this early stage (369, 370). Infusion of the ketone body D-β-hydroxybutyrate (BHB) and the antioxidant melatonin (MLT) in a low-volume resuscitation fluid increases survival in animal hemorrhagic shock models (231-233). BHB/MLT is safe and most effective when administered intravenously in the treatment of porcine hemorrhagic shock and polytrauma (293). In view of these promising preclinical results, optimization of the treatment formulation is an essential step in advancing BHB/MLT towards clinical use. We have previously shown that 4 M BHB/43 mM MLT is the optimal concentration for the treatment of porcine hemorrhagic shock, while in rat hemorrhagic shock, the melatonin concentration can be lowered without decreases in survival (233, 371).

Here, we describe the next step in the preclinical development and optimization of the BHB/MLT formulation as a treatment for hemorrhagic shock. An ideal intravenous formulation should fulfill multiple requirements: 1) adequate drug solubility for intravenous use, 2) acceptable clinical safety profile, 3) adequate chemical stability so

that the formulation can have a practically acceptable shelf life, and 4) fast and easy preparation for use. Due to the limited aqueous solubility of melatonin, the original BHB/MLT solution contains 20% v/v dimethyl sulfoxide (DMSO) as a cosolvent.

DMSO is beneficial in the treatment of hemorrhagic shock and cardiac and central nervous pathologies and hence may enhance the favorable effects of BHB/MLT (255-258, 276). DMSO alleviated hemorrhage-induced increases in the pro-inflammatory transcription factor NFκB and increased expression of the protective heat shock protein Hsp70 in the liver, kidney and intestine in rats (259). Hence, DMSO may contribute to the beneficial effects of BHB/MLT, an effect that could potentially be lost if it was removed. However, some properties of DMSO pose challenges in its clinical use. Infusion of DMSO, at concentrations exceeding 10%, can induce intravascular hemolysis. DMSO is also an emetic and a strong diuretic (246, 247, 263, 264). Combined, these effects can result in fluid loss and anemia, which may counteract the beneficial effects of BHB/MLT. Due to melatonin instability in aqueous solution (372), BHB/MLT has to be prepared immediately before use. The BHB/MLT/DMSO formulation requires a three-step reconstitution procedure, which poses a challenge when quick administration of the drug product is needed following traumatic injury.

Aqueous formulations containing BHB/MLT, wherein DMSO was replaced with alternative excipients, were tested for safety and efficacy in a lethal rat hemorrhagic shock model. Preliminary solubility studies revealed that the required aqueous concentration of melatonin could be obtained in (a) 20% w/v polyvinylpyrrolidone K12 (BHB/MLT/PVP) or (b) a mixture of 10% w/v hydroxypropyl-β-cyclodextrin, 5% w/v polyvinylpyrrolidone K12 and 5% w/v polyethylene glycol 400 (BHB/MLT/CD). One potential concern is the instability of melatonin in aqueous solutions. Lyophilization provided an avenue to prepare stable injectable formulations that can be reconstituted into solutions right before administration. We recently published our initial results of the *in vivo* efficacy of the BHB/MLT/PVP solution (373). Detailed characterization of 'as is' BHB and of BHB/MLT/PVP, in solution as well as in the lyophilized state, were conducted (373). Here, we present detailed analyses of the safety and efficacy of both BHB/MLT/PVP and BHB/MLT/CD in a rat model of hemorrhagic shock, along with comprehensive characterization of the BHB/MLT/CD prelyophilization solution and the final lyophile.

To summarize, we evaluated solutions of BHB/MLT containing DMSO, polyvinylpyrrolidone K12 (PVP) or a mix of hydroxypropyl-β-cyclodextrin (HPβCD), PVP and polyethylene glycol 400 (PEG), resulting in four treatment groups: (i) BHB (4 M)/MLT (43 mM)/DMSO (20% v/v). In the remaining formulations, the total solute concentration was reduced by a 1:1 dilution of the original 4 M BHB/43 mM MLT solution. While this doubled the injection volume, it remained substantially below the volume used for standard crystalloid resuscitation (31). (ii) BHB (2 M)/MLT (21.5 mM)/DMSO (10%), (iii) BHB/MLT/PVP and (iv) BHB/MLT/CD (Table 1). Treatment solutions were administered, in a preclinical hemorrhagic shock model, as intravenous boluses containing an equal dose of 4 mmol/kg BHB and 43 μmol/kg melatonin, a dose previously established to increase survival in rat hemorrhagic shock (231, 233).

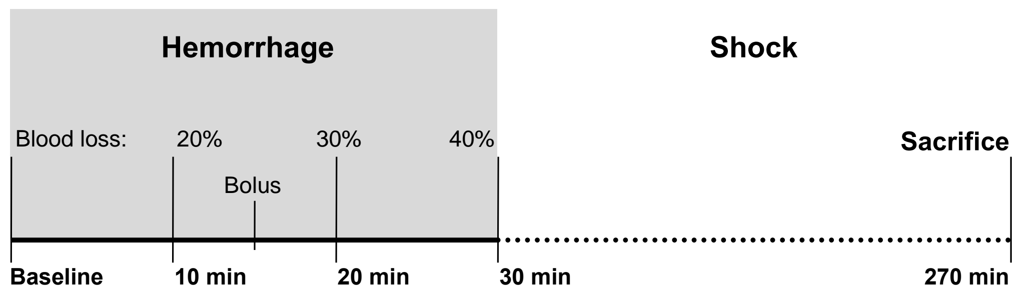


Figure 4.2. Hemorrhagic shock and infusion protocol. Treatment solutions were administered as a 1 ml/kg or 2 ml/kg bolus over 1 min.

Table 4.1. Treatment groups.

Treatment Group	[BHB]	[MLT]	[DMSO]	[PVP]	[HPβCD]	[PEG]	Volume	BHB/MLT Dose	n
Sham	-	-	-	-	-	-	-	-	10
No Treatment	-	-	-	-	-	-	-	-	11
LR	-	-	-	-	-	-	2 ml/kg	-	10
4 M BHB/MLT/DMSO	4 M	43 mM	20%	-	-	-	1 ml/kg	4 mmol BHB/kg 43 μmol MLT/kg	10
2 M BHB/MLT/DMSO	2 M	21.5 mM	10%	-	-	-	2 ml/kg		10
BHB/MLT/PVP	2 M	21.5 mM	-	10%	-	-	2 ml/kg		10
BHB/MLT/CD	2 M	21.5 mM	-	2.5%	5%	2.5%	2 ml/kg		10

Sham rats were exposed to instrumentation only, no bolus was administered and no blood was withdrawn other than for blood gas analysis at baseline and for blood gas and organ marker analysis at the end of the experiment. All treatments were delivered as an intravenous bolus over 1 minute.

Materials and methods

D-β-hydroxybutyrate was purchased from Sigma Aldrich (St. Louis, MO) and Lonza (Basel, Switzerland). Melatonin was generously provided by Flamma S.p.A. (Chignolo, Italy). DMSO was purchased from Alfa Aesar (Ward Hill, MA), PVP from Acros Organics (Geel, Belgium), and PEG from Spectrum Chemical (New Brunswick, New Jersey). HPβCD (molar substitution: 0.6) and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Solution preparation for Injection

BHB/MLT/DMSO solutions. On the day of the experiment, appropriate amounts of melatonin solution (DMSO as vehicle) and aqueous solution of BHB (pH 7.4) were mixed to achieve final solutions containing either BHB (4 M)/MLT (43 mM)/20% w/v DMSO or BHB (2 M)/MLT (21.5 mM)/10% w/v DMSO.

BHB/MLT/CD solution. Melatonin was dissolved in a solution containing 10% HPβCD/5% PVP/5% PEG. Equal volumes of 43 mM MLT solution and 4 M BHB (adjusted to pH 7.4) solution were mixed to prepare the BHB/MLT/CD solution. The solutions were filtered through a 0.2 μm syringe filter for sterilization before injection.

Hemorrhagic shock model

Anesthesia and instrumentation

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and carried out in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) regulations. Seventy-one male Sprague-Dawley rats (350-375 g, Envigo) were housed on a 12-hour light dark cycle with water and food *ad libitum*. The rats were allowed to adapt to their environment for at least seven days before the procedure. On the day of the experiment, rats were anesthetized in an induction chamber with isoflurane (5% in 1 l/min oxygen), treated with meloxicam for analgesia (0.5 mg, subcutaneous), and placed on a heating pad to maintain a target body temperature of 37 ± 0.5 ºC throughout the experiment (measured via rectal thermometer). Anesthesia was maintained with isoflurane via nose cone (1-2% in 1 l/min oxygen, Surgivet Isotec 4, Smiths Medical PM, Inc, Norwell, MA). The right external jugular vein was aseptically exposed and cannulated with sterile PE 160 tubing for blood drawing and treatment

administration. The left femoral artery was aseptically exposed and cannulated with a 24 G catheter for continuous heart rate and blood pressure measurements.

Hemorrhagic shock and infusion protocol

Instrumentation was followed by a stabilization period of 10 min, after which baseline measurements were taken. Immediately after baseline sampling, blood was gradually withdrawn in three steps to achieve a total loss of 40% of the total blood volume over 30 minutes (Figure 4.2). Total blood volume was calculated as 7% of body weight (374). Fourteen minutes after baseline measurements, half-way throughout blood withdrawal, treatments were administered as a 1 ml/kg or 2 ml/kg intravenous bolus over 1 minute (Table 4.1). Lines were flushed with lactated Ringer's solution (LR) after blood draws and solution administration. Animals that survived until the end of the experiment were maintained anesthetized until they were euthanized via exsanguination after the last sampling point (270 min). Sham rats were only instrumented, no treatment bolus was administered and no blood was withdrawn other than for blood gas analysis at baseline and for blood gas and organ marker analysis at the end of the experiment.

Hemodynamic and physiologic measurements

Blood pressure and heart rate were measured continuously throughout the experiment with a Spacelab monitor (Spacelabs Healthcare, Snoqualmie, WA). At baseline, at the end of blood removal (30 min) and at the end of the experiment (270 min), venous blood samples were drawn for analysis via blood gas analyzer (Gem Premier 3000, Instrumentation Laboratory Co, Bedford, MA) and to measure markers of organ function (including alkaline phosphatase (Alk Phos), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and blood urea nitrogen (BUN)). Free plasma hemoglobin, a marker of intravascular hemolysis, was measured photometrically at the end of the shock period (30 min). Organ function markers and free plasma hemoglobin were analyzed in the Clinical Laboratory Improvement Amendments-certified Fairview Diagnostics Laboratory.

Melatonin and BHB serum concentrations

BHB and melatonin serum concentrations were determined at baseline, at the end of the bolus infusion and immediately before sacrifice. Blood samples were allowed to clot at room temperature for 30 minutes before centrifugation (1150 g, 10 min, 4 ºC) and

collection of serum. Melatonin and BHB serum concentrations were measured as previously described in detail (293). In short, the samples were extracted with acetonitrile and dried under nitrogen. For melatonin quantification, the sample was dissolved in a 1:1 v/v mixture of water and methanol with 0.1% v/v formic acid. Melatonin was quantified via ultrahigh-performance liquid chromatography-coupled mass spectrometry (UPLC-MS; limit of quantitation $5x10^3$ pg/ml). For BHB analyses, samples were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide: pyridine (5:1) and then analyzed via gas chromatography-coupled mass spectrometry (GC-MS; limit of detection 0.048 mM).

In vitro hemolysis

In vitro hemolysis quantification was modified from Fort (375). Fifty µl of test solution and 450 µl of fresh heparinized human whole blood were gently mixed and incubated for 60 seconds, after which the solution was quenched with 10 ml isotonic PBS buffer. Isotonic PBS buffer was used as nonhemolytic control and 10% v/v Triton X 100 was used as positive control (376). Solutions were centrifuged (1500 g, 25 ºC, 15 min) and the supernatant was transferred to a clear 96-well plate (200 µl/well; in triplicate). Absorbance (Abs) was measured at 540 nm and hemolysis was calculated as (377):

$$\%\, Hemolysis = \left(\frac{Abs_{sample} - Abs_{PBS}}{Abs_{Triton\,X\,100} - Abs_{PBS}}\right) X\ 100$$

Initial experiments showed that, at the dilutions used in our experiment, none of the tested compounds changed the absorbance spectrum of hemoglobin (data not shown).

Lyophilization of the BHB/MLT/CD solution

BHB/MLT/CD solution was filled into 10 mL glass vials (2 mL fill volume), covered with rubber stoppers (20 mm, 2 Leg Lyo, Gry Butyl Sil, Wheaton), and loaded into the lyophilizer. Lyophilization was carried out in a bench top freeze-dryer (VirTis AdVantage, Gardiner, NY). The shelf was cooled to -60 °C at 0.25 °C/min and held for 8 h. Primary drying was sequentially conducted at -40 °C (12 h), -30 °C (24 h), and -20 °C (12 h) at 75 ± 25 mTorr. During secondary drying, the shelf was progressively heated to -10 °C, 0 °C, +10 °C and +25 °C, held at each temperature for 12 h and finally heated to 40 °C and held for 24 h. The samples were dried under vacuum for 48 h. At the end of the cycle, the vials were stoppered and stored in a desiccator containing anhydrous calcium sulfate at -20 °C.

Separate solutions of BHB (2 M; pH 7.4) and of MLT (21.5 mM) in 5 % w/v HPβCD, 2.5% w/v PVP and 2.5 % w/v PEG 400 were also prepared and lyophilized as above

Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Q2000, TA Instruments, New Castle, DE) equipped with a refrigerated cooling accessory was used. Dry nitrogen gas was purged at 50 mL/min. For thermal analysis of the prelyophilization solution, ~20 μL of solution was weighed in an aluminum pan, sealed hermetically, cooled from room temperature (RT) to -90 °C at 1 °C/min, held for 30 min and heated to RT at 10 °C/min. In case of lyophiles, the powder was filled into the aluminum pan at RT (in a glove box under nitrogen purge; RH ≤ 5%), sealed non-hermetically, and heated from RT to 180 °C, at 10 °C/min.

X-ray diffractometry (XRD)

Powder samples were exposed, at room temperature, to Co Kα radiation (1.78899 Å; 40 kV × 35 mA) in a two-dimensional X-ray diffractometer (D8-Discover fitted with a Vantec 500 detector, Bruker). XRD patterns were collected using a 0.8 mm collimator, set at a 10° angle of incidence and an area detector (angular range 35°) set at an angle of diffraction of 20° 2θ. Area detector images were finally converted to one-dimensional intensity vs. 2θ data sets by using an averaging integration algorithm.

Statistical analysis

Kaplan-Meier analysis with Generalized Wilcoxon test was used to analyze survival differences between groups. Non-longitudinal data were analyzed via Kruskal-Wallis test with Dunn-Bonferroni corrections and are reported as medians with interquartile ranges (IQR). Longitudinal parameters were analyzed using the Proc Mixed procedure in SAS Version 9.4 software (SAS Institute, Inc., Cary, NC). Group, Time and Group*Time Interaction were modeled as fixed effects. The models used compound symmetry or autoregressive covariance structure (selection based on the model with the lowest Bayesian Information Criterion value) and the between-within method for degrees of freedom. For parameters with significant interaction effects, differences at individual time points were analyzed by pairwise comparisons with Tukey adjustments. Longitudinal data is presented as least-squares means with 95% confidence intervals. Least squares means are the predicted population means of fixed effects over a balanced

population, with the standard errors adjusted for the covariance parameters. A confidence interval (CI) is a range of values that is likely to contain the true population mean. If a population is repeatedly tested, and each time a 95% CI is estimated, 95% of these intervals will include the true mean.

Results

Physiology

Mean arterial pressure (MAP) and heart rate were measured throughout the experiment (Figure 4.3). Rats exposed to hemorrhage experienced a sharp drop in MAP upon the beginning of blood removal (Figure 4.3 A). Infusion of BHB/MLT, but not LR transiently increased MAP. However, MAP was similar in all hemorrhaged groups at the end of the blood removal phase. The MAP increased once blood withdrawal was completed but remained below baseline levels until the end of the experiment. MAP remained stable in the sham group and was significantly higher than in all hemorrhaged groups during the first three hours after the completion of blood withdrawal.

Although there was a significant interaction effect for heart rate, there were no significant differences between groups at individual time points (Figure 4.3 B). Heart rate decreased after the first blood draw and then increased throughout the experiment, an effect that was most prominent in the BHB/MLT–treated groups. We observed no significant group or interaction effects for body temperature (G $p = 0.8587$, T $p < 0.0001$, G*T $p = 0.2916$).

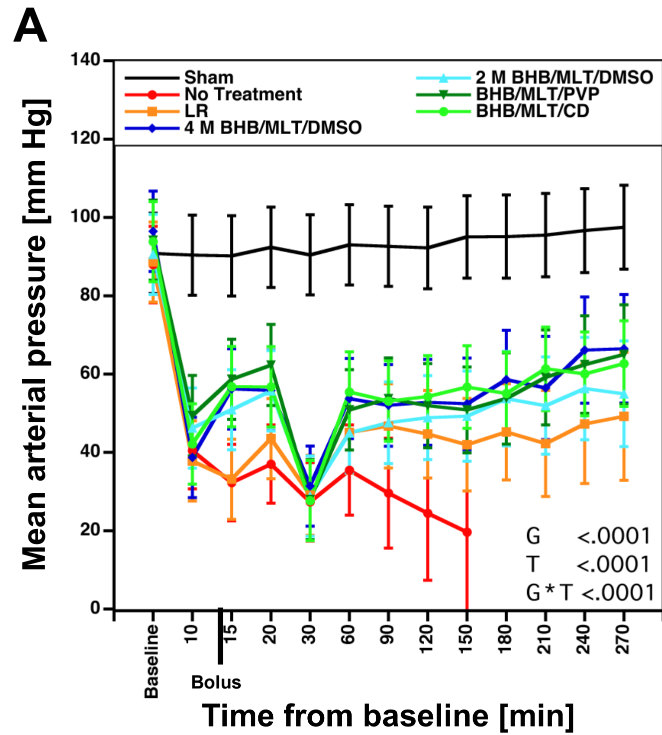
Survival

All sham animals survived until the end of the experiment, while all rats exposed to blood loss without treatment died within three hours of baseline measurements (Figure 4.4). The highest survival was observed in rats treated with BHB/MLT/CD (8/10), followed by rats receiving BHB/MLT/PVP (6/10), 4 M BHB/MLT/DMSO (5/10) and 2 M BHB/MLT/DMSO (5/10) and those treated with LR (3/10). Survival was significantly lower in untreated rats than in the LR and each of the BHB/MLT groups ($p \leqq 0.002$). There was no significant difference in survival between the BHB/MLT-treated groups. Survival in BHB/MLT/CD-treated rats was not significantly different from the sham group ($p = 0.147$), and was significantly higher when compared to the LR group ($p = 0.018$).

Drug serum concentrations

To test the effects of solution formulation on systemic drug levels, we determined the serum concentrations of melatonin and BHB at baseline, after bolus infusion and at the end of the experiment. BHB and melatonin serum concentrations peaked after bolus

infusion and returned close to baseline levels by the end of the experiment in BHB/MLT-infused animals. Drug serum concentrations remained at baseline levels in untreated rats and in those receiving LR (Figure 4.5). After bolus infusion, BHB and melatonin levels were significantly higher in the groups receiving solutions containing BHB/MLT than in untreated rats or those infused with LR. BHB serum levels were significantly higher in the BHB/MLT/PVP than in the 2 M BHB/MLT/DMSO group after the bolus infusion (Figure 4.5 A, $p=0.0283$). Melatonin serum levels did not differ significantly between the BHB/MLT-treated groups (Figure 4.5 B).



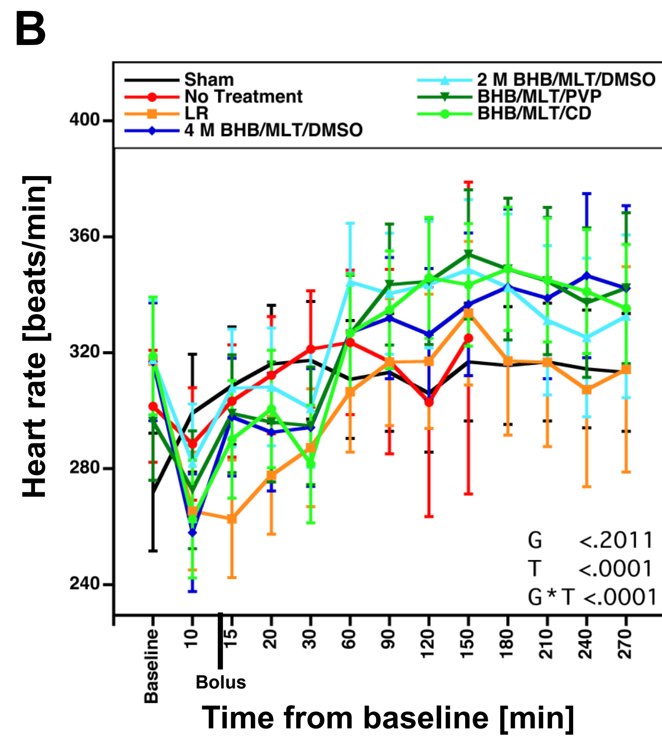


Figure 4.3. (A) Mean arterial pressure and (B) heart rate as a function of time in rats exposed to 40% blood loss and treated with LR or different formulations of BHB/MLT. Data are presented as least squares means with 95% CIs.

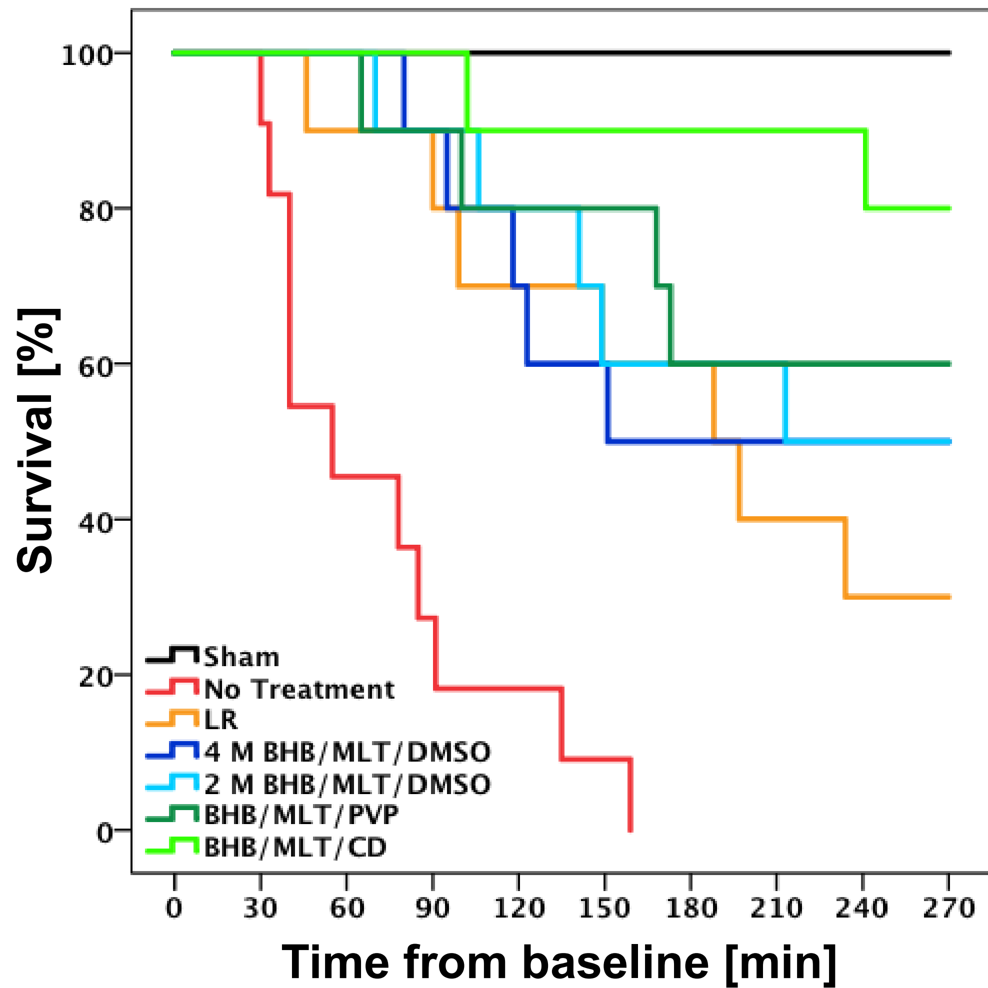


Figure 4.4. Kaplan-Meier survival curve of rats exposed to 40% blood loss and treated with LR or different formulations of BHB/MLT.

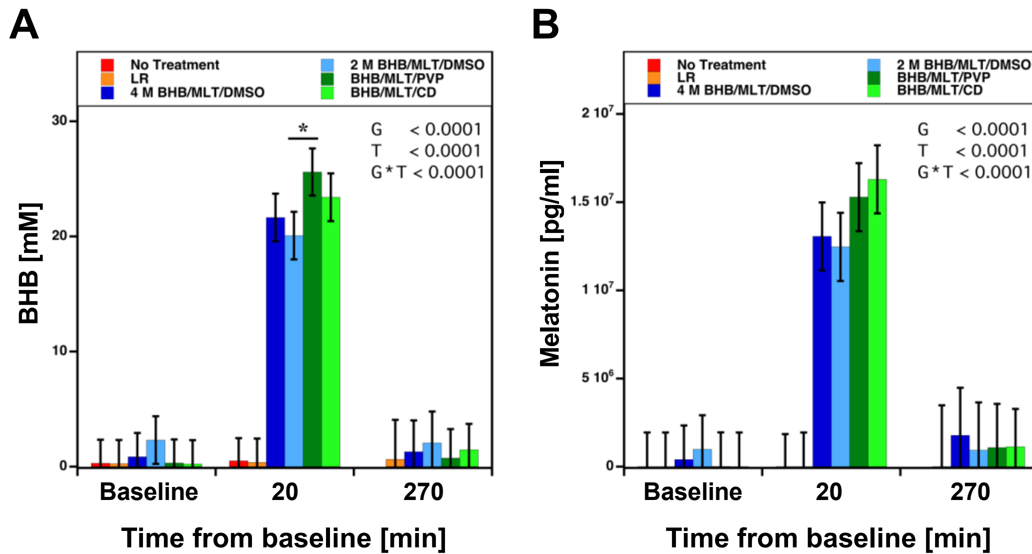


Figure 4.5. Serum concentrations of (A) BHB and (B) melatonin at select time points in rats subjected to 40% blood loss and treated with LR or different formulations of BHB/MLT. Data are presented as least squares means with 95% CIs. * $p<0.05$ for BHB/MLT/PVP vs 2 M BHB/MLT/DMSO.

Blood gases and markers of organ function

Blood gases and various markers of organ function were analyzed at baseline, at the end of blood withdrawal and at the end of the experiment (Table 4.2). Changes in blood electrolytes in BHB/MLT treated rats were similar to those previously observed after the infusion of BHB and melatonin in porcine hemorrhagic shock (232, 293). BHB/MLT administration resulted in significantly increased blood sodium concentrations at the end of blood withdrawal (compared to LR). This was likely a direct effect of the BHB/MLT infusion, which is administered as a sodium salt. BHB/MLT and LR infusion attenuated a shock-induced (i) increase in blood lactate levels, (ii) decrease in blood pH and (iii) decrease in base excess. These effects were more pronounced after BHB/MLT treatment.

Total hemoglobin levels were significantly decreased in hemorrhaged animals at the end of the experiment. As expected, there was no treatment effect, with either LR or with BHB/MLT. Infusion of BHB/MLT, but not LR, attenuated a hemorrhage-induced decrease in venous oxygen saturation.

Table 4.2. Blood Gases and markers of organ function in Rats Exposed to 40% blood loss and treated with LR or different formulations of BHB/M.

Parameter	Baseline	S 30 min	S 270 min	Effects
Na^+ [mEq/l]				
Sham	138.2 (135.4-141.0)		138.4 (135.7-141.2)	G .0588
No Treatment	137.2 (134.6-139.8)	134.5 (131.9-137.2)		T <.0001
LR	134.0 (131.4-136.7)	132.2 (129.4-134.9)	137.8 (132.9-142.7)	G*T .0138
4 M BHB/MLT/DMSO	137.6 (135.0-140.3)	139.2 (136.6-141.9)$^{\#}$	139.6 (136.3-143.0)	
2 M BHB/MLT/DMSO	138.0 (135.4-140.7)	139.7 (136.9-142.4)$^{\#}$	143.4 (140.0-146.8)	
BHB/MLT/PVP	135.5 (132.9-138.2)	138.3 (135.7-141.0)	140.3 (137.1-143.4)	
BHB/MLT/CD	137.0 (134.0-140.0)	139.9 (136.9-142.8)$^{\#}$	141.5 (138.4-144.6)	
K^+ [mEq/l]				
Sham	4.34 (3.64-5.05)		5.17 (4.47-5.87)	G .0055
No Treatment	4.72 (4.06-5.39)	6.02 (5.36-6.69)		T<.0001
LR	4.17 (3.51-4.84)	5.25 (4.55-5.95)	6.60 (5.12-8.09)	G*T .0689
4 M BHB/MLT/DMSO	4.34 (3.68-5.01)	4.45 (3.79-5.12)	5.02 (4.08-5.96)	
2 M BHB/MLT/DMSO	4.38 (3.72-5.05)	4.23 (3.53-4.93)	6.43 (5.49-7.37)	
BHB/MLT/PVP	4.28 (3.62-4.95)	4.28 (3.62-4.95)	6.18 (5.32-7.04)	
BHB/MLT/CD	4.30 (3.56-5.04)	4.10 (3.36-4.84)	5.14 (4.35-5.94)	
pH				
Sham	7.32 (7.26-7.38)		7.24 (7.17-7.30)	G <.0001
No Treatment	7.31 (7.25-7.37)	7.06 (7.00-7.12)		T <.0001
LR	7.36 (7.29-7.42)	7.18 (7.12-7.25)	7.12 (6.99-7.25)	G*T <.0001
4 M BHB/MLT/DMSO	7.37 (7.31-7.43)	7.35 (7.28-7.41)$^{\wedge}$	7.37 (7.28-7.45)	
2 M BHB/MLT/DMSO	7.35 (7.29-7.41)	7.35 (7.28-7.41)$^{\wedge}$	7.20-(7.12-7.29)	
BHB/MLT/PVP	7.35-(7.29-7.41)	7.35 (7.29-7.41)$^{\wedge\#}$	7.33 (7.25-7.41)	
BHB/MLT/CD	7.36 (7.29-7.43)	7.36 (7.29-7.43)$^{\wedge\#}$	7.35 (7.28-7.43)	

Parameter	Baseline	S 30 min	S 270 min	Effects
Base Excess				
Sham	1.3 (-1.0-3.7)		0.5 (-1.9-2.8)	G <.0001
No Treatment	2.4 (0.2-4.6)	-8.1 (-10.5- -5.8)		T <.0001
LR	3.6 (1.4-5.9)	-4.7 (-7.1- -2.4)	-4.6 (-9.5-0.3)	G*T <.0001
4 M BHB/MLT/DMSO	3.2 (1.0-5.4)	2.4 (0.2-4.6)$^{\wedge\#}$	4.2 (1.0-7.3)	
2 M BHB/MLT/DMSO	2.7 (0.5-5.0)	2.8 (0.5-5.2)$^{\wedge\#}$	4.7 (1.2-8.1)	
BHB/MLT/PVP	3.4 (1.1-5.6)	2.9 (0.7-5.1)$^{\wedge\#}$	2.3 (-0.5-5.2)	
BHB/MLT/CD	4.7 (2.2-7.2)	3.4 (0.9-5.9)$^{\wedge\#}$	3.9 (1.2-6.5)	
Lactate [mg/dl]				
Sham	3.3 (1.9-4.7)		2.0 (0.6-3.4)	G .0006
No Treatment	2.0 (0.7-3.4)	9.4 (8.0-10.7)		T <.0001
LR	1.8 (0.5-3.1)	6.6 (5.2-8.0)	2.1 (-0.9-5.1)	G*T <.0001
4 M BHB/MLT/DMSO	1.9 (0.5-3.2)	3.7 (2.4-5.1)$^{\wedge}$	3.6 (1.8-5.5)	
2 M BHB/MLT/DMSO	1.8 (0.5-3.1)	3.3 (1.8-4.7)$^{\wedge}$	6.3 (4.4-8.2)*	
BHB/MLT/PVP	2.0 (0.7-3.3)	3.6 (2.3-4.9)$^{\wedge}$	4.6 (2.8-6.3)	
BHB/MLT/CD	1.2 (-0.3-2.7)	2.8 (1.3-4.3)$^{\wedge\#}$	3.7 (2.1-5.3)	
Glucose [mg/dl]				
Sham	316 (268-364)		202 (154-250)	G .2353
No Treatment	353 (308-399)	483 (437-528)		T <.0001
LR	316 (270-361)	475 (427-523)	116 (15-217)	G*T .7572
4 M BHB/MLT/DMSO	306 (261-352)	457 (411-502)	254 (190-318)	
2 M BHB/MLT/DMSO	324 (278-369)	466 (418-514)	229 (165-293)	
BHB/MLT/PVP	318 (273-364)	449 (403-494)	209 (151-268)	
BHB/MLT/CD	290 (239-341)	424 (373-475)	190 (136-244)	

Parameter	Baseline	S 30 min	S 270 min	Effects
Ca^{2+} [mEq/l]				
Sham	1.16 (1.07-1.25)		1.23 (1.14-1.33)	G .2888
No Treatment	1.15 (1.07-1.24)	1.17 (1.08-1.26)		T .0010
LR	1.17 (1.08-1.25)	1.11 (1.02-1.21)	1.00 (0.83-1.18)	G*T .0421
4 M BHB/MLT/DMSO	1.14 (1.06-1.23)	1.09 (1.00-1.17)	1.03 (0.91-1.15)	
2 M BHB/MLT/DMSO	1.18 (1.09-1.26)	1.09 (1.00-1.18)	1.15 (1.04-1.27)	
BHB/MLT/PVP	1.26 (1.17-1.35)	1.13 (1.04-1.22)	1.07 (0.96-1.18)	
BHB/MLT/CD	1.17 (1.07-1.26)	1.04 (0.94-1.14)	1.01 (0.91-1.12)	
Hemoglobin [g/dl]				
Sham	11.8 (11.1-12.6)		11.0 (10.2-11.7)	G <.0005
No Treatment	11.7 (11.0-12.4)	8.1 (7.4-8.8)		T <.0001
LR	12.0 (11.3-12.7)	7.6 (6.9-8.4)	6.3 (4.9-7.6)*	G*T .0001
4 M BHB/MLT/DMSO	12.6 (11.9-13.3)	8.5 (7.8-9.2)	6.8 (5.9-7.7)*	
2 M BHB/MLT/DMSO	12.6 (11.8-13.3)	8.0 (7.3-8.8)	6.8 (5.9-7.8)*	
BHB/MLT/PVP	12.2 (11.5-12.9)	8.1 (7.4-8.8)	7.0 (6.1-7.8)*	
BHB/MLT/CD	12.5 (11.7-13.2)	8.0 (7.2-8.8)	6.6 (5.8-7.4)*	
SvO_2 [%]				
Sham	88.6 (79.1-98.0)		85.9 (76.5-95.3)	G .0001
No Treatment	77.3 (68.4-86.2)	19.1 (9.8-28.5)		T <.0001
LR	86.7 (77.8-95.6)	29.5 (20.1-38.9)	56.2 (37.4-75.0)	G*T .0012
4 M BHB/MLT/DMSO	88.8 (79.9-97.7)	49.2 (40.3-58.1)	64.0 (51.8-76.1)	
2 M BHB/MLT/DMSO	87.8 (78.9-96.7)	48.8 (39.4-58.2)^	66.5 (53.0-80.1)	
BHB/MLT/PVP	86.7 (77.8-95.6)	53.9 (45.0-62.8)^#	63.5 (52.3-74.7)	
BHB/MLT/CD	87.4 (77.4-97.4)	51.0 (41.0-61.0)^	68.9 (58.3-79.5)	

Parameter	Baseline	S 30 min	S 270 min	Effects
ALT [U/dl]				
Sham			46 (-166-258)	G .0387
No Treatment	46 (-156-248)	41 (-161-243)		T .0001
LR	50 (-162-262)	33 (-179-245)	158 (-229-545)	G*T .3062
4 M BHB/MLT/DMSO	48 (-176-271)	39 (-173-251)	128 (-172-428)	
2 M BHB/MLT/DMSO	44 (-168-256)	32 (-180-244)	768 (468-1068)	
BHB/MLT/PVP	49 (-163-261)	33 (-179-245)	584 (310-857)	
BHB/MLT/CD	47 (-190-284)	33 (-190-256)	286 (49-523)	
AST [U/dl]				
Sham			85 (-150-319)	G .0230
No Treatment	70 (-154-293)	82 (-142-305)		T <.0001
LR	76 (-159-310)	63 (-171-298)	322 (-106-750)	G*T .2620
4 M BHB/MLT/DMSO	76 (-171-323)	79 (-168-327)	349 (17-680)	
2 M BHB/MLT/DMSO	68 (-179-316)	59 (-176-293)	143 (-189-475)	
BHB/MLT/PVP	77 (-185-339)	68 (-167-302)	943 (640-1245)	
BHB/MLT/CD	75 (-187-337)	59 (-188-306)	609 (346-871)	
Alk Phos [U/dl]				
Sham			123 (107-139)	G .3601
No Treatment	128 (112-143)	107 (92-122)		T <.0001
LR	123 (107-139)	90 (74-106)	116 (89-144)	G*T .8087
4 M BHB/MLT/DMSO	130 (114-147)	98 (81-114)	113 (92-135)	
2 M BHB/MLT/DMSO	107 (91-123)	95 (78-111)	97 (76-119)	
BHB/MLT/PVP	122 (106-138)	88 (72-104)	110 (90-130)	
BHB/MLT/CD	121 (103-138)	91 (74-108)	112 (94-129)	

Parameter	Baseline	S 30 min	S 270 min	Effects
BUN [U/dl]				
Sham			31.7 (29.2-34.2)	G <.0001
No Treatment	20.4 (18.0-22.7)	21.1 (18.7-23.5)		T .0001
LR	17.0 (14.5-19.5)	19.3 (16.8-21.8)	41.8 (37.4-46.3)	G*T .1334
4 M BHB/MLT/DMSO	18.0 (15.4-20.6)	22.9 (20.4-25.4)	40.2 (36.7-43.7)	
2 M BHB/MLT/DMSO	14.2 (11.7-16.7)	20.1 (17.6-22.6)	45.1 (41.6-48.6)	
BHB/MLT/PVP	17.1 (14.6-19.6)	19.7 (17.2-22.2)	40.7 (37.6-43.9)	
BHB/MLT/CD	17.3 (14.5-20.1)	20.0 (17.4-22.6)	43.7 (41.0-46.5)	
Creatinine [U/dl]				
Sham			0.37 (0.26-0.48)	G <.0001
No Treatment	0.39 (0.29-0.49)	0.49 (0.39-0.59)		T <.0001
LR	0.27 (0.16-0.38)	0.47 (0.37-0.58)	1.30 (1.10-1.50)	G*T .1766
4 M BHB/MLT/DMSO	0.26 (0.15-0.38)	0.48 (0.37-0.59)	0.98 (0.83-1.13)	
2 M BHB/MLT/DMSO	0.30 (0.19-0.40)	0.39 (0.28-0.50)	0.91 (0.75-1.06)	
BHB/MLT/PVP	0.25 (0.15-0.36)	0.40 (0.29-0.50)	0.84 (0.70-0.98)	
BHB/MLT/CD	0.27 (0.15-0.39)	0.41 (0.30-0.52)	1.00 (0.88-1.12)	

Data are presented as least-squared means (95% confidence interval). [*] p<0.05 versus Sham at the same time point, [^] versus No Treatment at the same time point, [#] versus LR at the same time point.

We observed hemorrhage-induced increases in serum levels of various markers of liver (ALT, AST) and kidney function (BUN and creatinine) at the end of the experiment, independent of treatment group. Alk Phos did not change significantly throughout the experiment. Blood glucose and calcium concentrations did not differ significantly between groups at individual time points. There were no significant differences between the four BHB/MLT formulations in their effect on the blood markers tested throughout the experiment.

Hemolysis

Hemolysis inducing-potential of the BHB/MLT formulations was evaluated both *in vitro* and *in vivo* (Figure 4.6). To account for the effects of solution tonicity, we analyzed BHB/MLT solutions containing DMSO, PVP or HPβCD/PVP/PEG at two concentrations *in vitro*. At a concentration of 4 M BHB/43 mM MLT, all three BHB/MLT solutions induced significantly more hemolysis than LR (Figure 4.6 A). When the solution concentration was lowered to 2 M BHB/21.5 mM MLT, the effect decreased and only the solution containing DMSO induced significantly more hemolysis than LR (Figure 4.6 B). The formulations with DMSO consistently induced the highest level of hemolysis, although differences between the three BHB/MLT formulations were not statistically significant. To evaluate formulation effects on hemolysis-induction *in vivo*, we measured free plasma hemoglobin at the end of blood withdrawal (Figure 4.6 C). The trend was similar to that observed *in vitro*: free plasma hemoglobin was highest after treatment with the 4 M BHB/MLT/DMSO solution, followed by 2 M BHB/MLT/DMSO. Free plasma hemoglobin in rats treated with BHB/MLT/PVP or BHB/MLT/CD were comparable to those in rats receiving LR, and significantly lower than in the BHB/MLT/DMSO groups.

Though limited in scope, the lethal rat hemorrhagic shock model studies revealed that both BHB/MLT/PVP and BHB/MLT/CD were effective. The efficacy of the BHB/MLT/CD solution exceeded that of the original DMSO-based formulation. The removal of DMSO resulted in significantly reduced *in vivo* hemolysis. This was evident with both BHB/MLT/PVP and BHB/MLT/CD-treated rats. However, the other indicators of toxicity did not differ between different formulations.

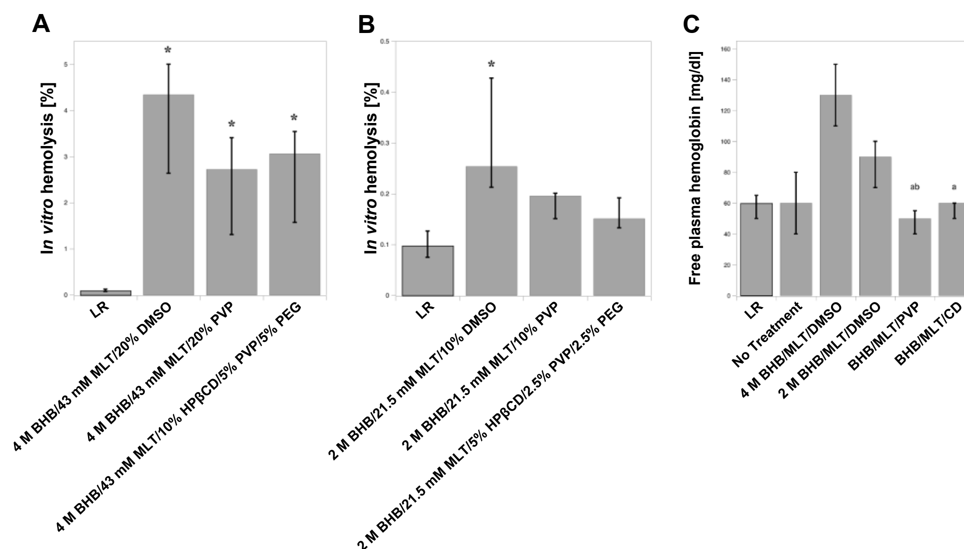


Figure 4.6. (A, B) *In vitro* and (C) *in vivo* hemolysis induction of the different BHB/MLT formulations. The BHB, MLT and excipient concentrations in (B) were half of that in (A). (C) Free plasma hemoglobin concentration was the marker of *in vivo* hemolysis. Data are presented as medians with interquartile range. * $p<0.05$ vs LR, a $p<0.05$ vs 4 M BHB/43 mM MLT/20% DMSO, b $p<0.05$ vs 2 M BHB/21.5 mM MLT/10% DMSO.

Development of lyophilized formulations

Since melatonin lacks adequate chemical stability to be formulated as a ready-to-use solution, lyophilization provides an avenue to prepare a stable injectable formulation. In an effort to prepare lyophiles with desirable attributes, such as low water content and short reconstitution times, lyophilization cycle process parameters were optimized. Thermal analyses of prelyophilization solutions enabled the selection of the lyophilization process parameters. The design and development of the PVP based formulation was the subject of an earlier publication (373).

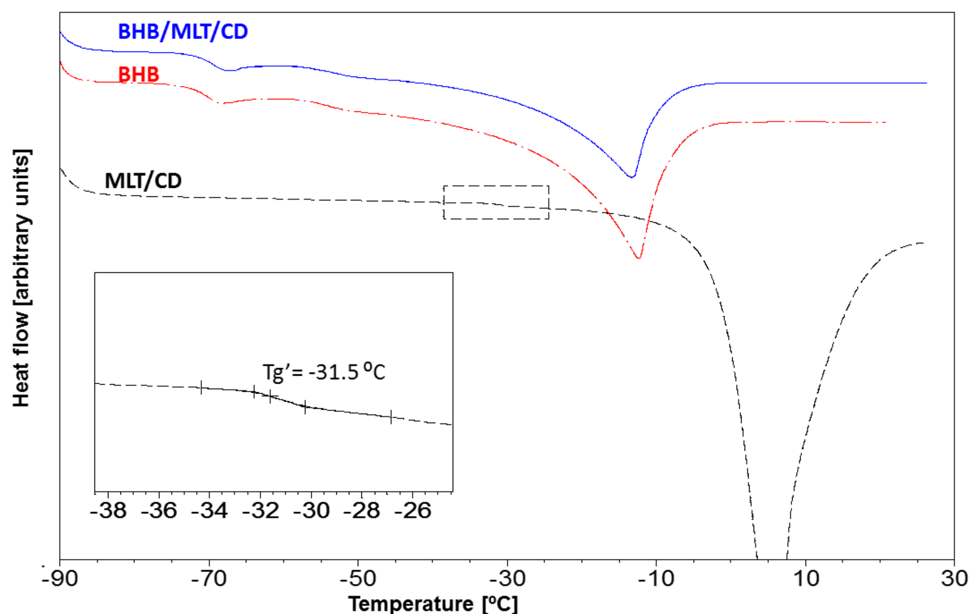


Figure 4.7. Differential scanning calorimetry (DSC) heating curve of frozen aqueous solutions of MLT/CD, BHB/MLT/CD and BHB. The solutions were initially cooled from RT to -90 °C at 1 °C/min, held for 30 min and heated to RT at 10 °C/min. Only the heating curves are shown. In the inset, a select region has been expanded to enable visualization of glass transition of MLT/CD freeze-concentrate (Tg′).

Characterization of prelyophilization solution

The DSC heating curves of frozen solutions of MLT/CD, BHB/MLT/CD and BHB are presented in Figure 4.7. The frozen MLT/CD solution exhibited a baseline shift at ~ −31 °C, attributed to Tg' (glass transition temperature of the freeze concentrate), followed by an endotherm at ~ 3 °C due to ice melting. The DSC heating curve of frozen BHB (alone) or BHB/MLT/CD solution exhibited two baseline shifts, at ~ -69 °C (Tg'') and at ~-53 °C (Tg'), followed by an endotherm at ~-13 °C, likely attributable to ice melting. Frozen solutions of several compounds of pharmaceutical interest, including mannitol, sucrose and trehalose, exhibit multiple glass transitions (378, 379). In these systems, the higher glass transition temperature (typically referred to as Tg') is considered the "true" glass transition of the maximally freeze-concentrated amorphous phase. A second phase, containing more water than the maximally freeze concentrated phase, exhibits a glass transition at a lower temperature (Tg"). When a BHB solution was

cooled, the solute crystallized (373). However, BHB crystallization was inhibited in presence of PVP (373). In the current system containing HPβCD and PVP, both noncrystallizing solutes, BHB crystallization was inhibited (data not shown). Since BHB was crystalline in the final lyophile, its crystallization would have occurred only during the drying stage of the lyophilization cycle (see results in the lyophile characterization studies).

Freeze drying cycle – development and optimization

Our initial freeze-drying cycle (fast freezing using liquid nitrogen, primary drying initiated at -25 °C; final secondary drying at 25 °C) yielded a lyophile with an unacceptably long reconstitution time of ~ 30 minutes. Therefore, we modified the process parameters by employing a conservative lyocycle, similar to the one used for lyophilization of sugars and hydrophilic polymers (380-382). The low glass transition temperature of the BHB/MLT/CD prelyophilization solution necessitated primary drying to be initiated at a low temperature of -40 °C (75 mTorr). The shelf temperature was sequentially increased, with the final drying at +40 °C (details in the Materials and Methods section). The hygroscopic nature of PVP and PEG dictated terminal drying to be conducted at a high temperature, followed by vacuum drying. This optimized lyophilization cycle yielded a BHB/MLT/CD lyophile with a reconstitution time of ~ 3 min.

Lyophile characterization

Earlier, based on extensive characterization studies, we had established that the ‘as is’ BHB existed as a hydrate (BHB·0.25 H_2O) under ambient conditions (373). The XRD patterns of the final BHB/MLT/CD lyophile and the ‘as is’ BHB (Figure 4.8), are virtually superimposable, suggesting that BHB crystallized as BHB·0.25 H_2O in the lyophile. No characteristic peaks of melatonin were discernible. Since PVP and HPβCD are amorphous and PEG 400 is a liquid at room temperature, there were no characteristic crystalline peaks attributable to these compounds. The amorphous nature of MLT/CD lyophile was evident from the XRD pattern, which was a broad halo.

The DSC heating curve of BHB (Figure 4.9 C) revealed a broad endotherm over 80-130 °C, attributed to dehydration followed by the vaporization of water. There were two additional thermal events at higher temperatures and these were discussed in our earlier

publication (373). The BHB/MLT/CD lyophile exhibited one broad endotherm from 80 to 120 °C, which can be attributed to dehydration (of BHB hydrate) followed by the vaporization of water. The thermal events observed at temperatures higher than 120 °C were not investigated. The lyophile containing MLT/CD (Figure 4.9 A) exhibited no discernible thermal events.

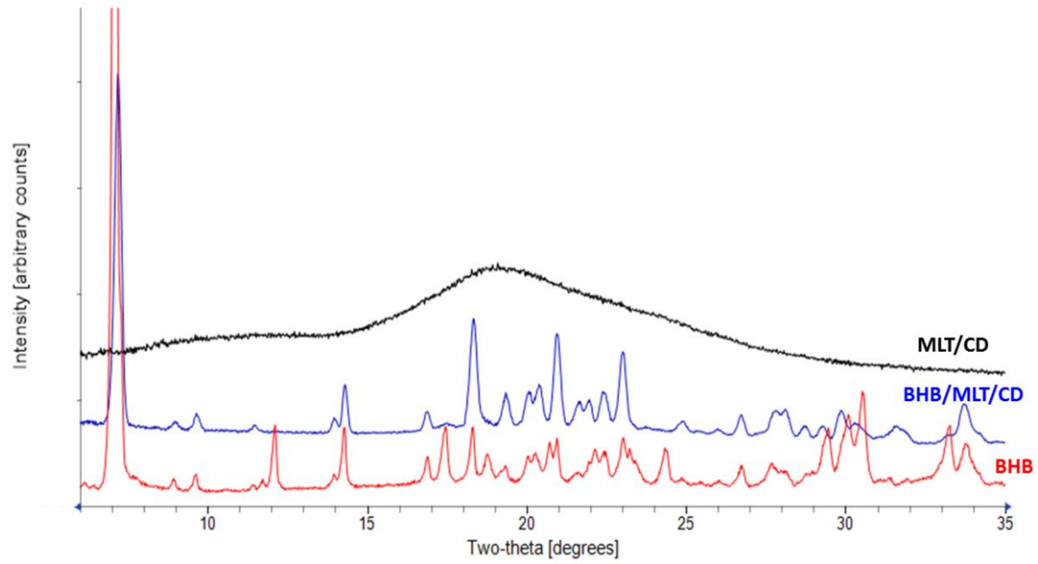


Figure 4.8. X-ray diffraction (XRD) patterns of MLT/CD lyophile, BHB/MLT/CD lyophile and BHB 'as is'.

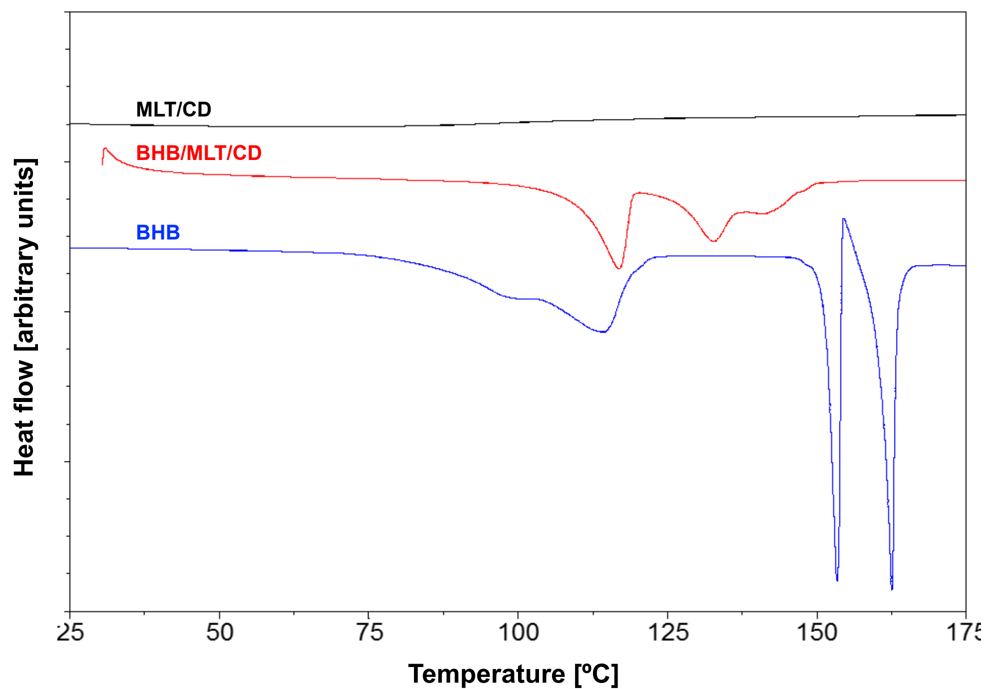


Figure 4.9. Differential scanning calorimetry (DSC) heating curves of MLT/CD lyophile, 'as is' BHB and BHB/MLT/CD lyophile.

Discussion

Intravenous infusion of 4 M BHB/43 mM MLT/20% DMSO, the formulation currently in preclinical testing, significantly improves survival in both rat and pig hemorrhagic shock models without inducing ongoing adverse effects (231, 232, 293). Melatonin is unstable in aqueous solution with a half-life of 33 days at 20 °C (372). Therefore, the infusion is prepared immediately before use. Another potential limitation is that the preparation process requires multiple steps. Such a complex preparation procedure, coupled with the requirement for aseptic processing, can be challenging for first responders in the field.

As a treatment for acute hemorrhagic shock, BHB/MLT is envisioned to be administered at the scene or during transport to specialized care. It was therefore desirable that the final formulation withstands storage under extreme conditions. Lyophilization is commonly used to prepare formulations of active ingredients which when formulated as aqueous solutions do not exhibit long term stability (e.g. melatonin). Furthermore, sterile (membrane) filtration of the prelyophilization solution, followed by freeze-drying under aseptic conditions, yields sterile formulations and obviates the need for terminal sterilization.

The goal of the current study was to formulate, prepare and evaluate a solid BHB/MLT dosage form which is: a) safe and effective, b) freeze-dried to enhance stability and c) can be readily reconstituted without loss of sterility. We identified two aqueous solutions with adequate melatonin solubility: 2 M BHB/21.5 mM MLT in (i) 10% PVP (BHB/MLT/PVP) and (ii) 5% HPβCD/2.5% PVP/2.5% PEG (BHB/MLT/CD, Table 1). The prelyophilization solutions were subjected to freeze-drying yielding solid lyophiles. The formulations were reconstituted, in less than 3 minutes, into an injection ready for administration.

The excipients employed in the novel BHB/M formulations were selected based on their favorable clinical safety profiles. HPβCD is used for solubility enhancement in multiple parenteral formulations and is well tolerated in humans (383-386). PVP exhibits very low acute toxicity (387), and high molecular weight PVP (K30) was widely used as a plasma expander during the second world war, and has not been associated with acute adverse effects (388). However, infusion of large amounts (>70 g) of high molecular

weight PVP can lead to “PVP storage disease”, wherein it accumulates in organs of the reticuloendothelial system (387, 389). The average molecular weight of PVP K12 used in our study (3,500 g/mol) is well below the limit for retention (390). Polyethylene glycols are used as cosolvents in several intravenous dosage forms and are generally regarded to have low toxicity (391). Some cases of renal necrosis were reported in humans after intravenous PEG 400 infusion. These occurred only after repeated administration of high doses (>120 g PEG 400, (392)).

Modification of the excipient composition can result in changes in efficacy and safety of medications. To be suitable for clinical use, non-DMSO formulations of BHB/MLT must at least retain the survival benefit observed with the original 4 M BHB/43 mM MLT/20% DMSO formulation. We evaluated the effects of BHB/MLT/DMSO, BHB/MLT/PVP and BHB/MLT/CD solutions on survival, intravascular hemolysis and various markers of organ injury in a rat hemorrhagic shock model. Survival was not significantly different between the four BHB/MLT-treated groups (Figure 4.4). Furthermore, in the BHB/MLT/CD group, survival was comparable to that in the sham group and significantly higher than in LR-treated controls. Efficacy was unaffected when the original formulation was administered as a 1:1 dilution, suggesting that the increased efficacy of BHB/MLT/CD was not due to increased infusion volume. Combined, our results suggest that a) the survival benefit of BHB/MLT-treatment after hemorrhagic shock is retained when DMSO is replaced with aqueous PVP solution, and b) BHB/MLT/CD exhibits increased efficacy when compared to the original formulation in this preclinical model of hemorrhagic shock. Hence, both novel BHB/MLT formulations constitute promising candidates for clinical use of the treatment.

Peak drug serum concentrations did not differ significantly between BHB/MLT/CD and the other BHB/MLT groups (Figure 4.5). To evaluate the systemic toxicity of the different BHB/MLT formulations, we evaluated various markers of physiological function and organ injury (Table 4.2). There were no significant differences between the four BHB/MLT groups, suggesting a similar safety profile for all of the tested BHB/MLT formulations. However, our data indicates that elimination of DMSO in BHB/MLT solutions is associated with decreased induction of hemolysis both *in vitro* and *in vivo* (Figure 4.6). In intravascular hemolysis, injury of the cellular membrane leads to the release of hemoglobin from erythrocytes into the plasma, resulting in decreased oxygen

transport and other adverse effects, which can include pain, vascular irritation, anemia, jaundice, acute renal failure and death (377, 393). These effects constitute a potential risk for the treatment of severe blood loss, which comes with an inherent decrease in oxygen transport and a risk for multiple organ failure (5).

Increased hemolysis after BHB/MLT/DMSO treatment was not unexpected, since infusion of solutions containing 10% v/v DMSO caused hemolysis and hemoglobinuria, an effect that was more pronounced with higher DMSO concentrations (245, 247, 263, 276, 308, 394-397). In contrast, complexation of low-solubility drugs with HPβCD or PVP of varying molecular weights significantly decreased hemolysis *in vitro* and *in vivo* (398-400). Furthermore, adding PEG 400 to solutions containing another cosolvent (e.g. DMSO) had a protective effect on red blood cells (401). In our study, differences in *in vivo* hemolysis were not associated with significant changes in concentrations of hemoglobin or markers of kidney function (Table 4.2). This suggests that the hemolysis induced in our experiment did not induce severe anemia or kidney injury. Indeed, DMSO-induced hemolysis and hemoglobinuria are commonly transient and not associated with changes in renal function (247, 263, 276, 394).

The improved efficacy of BHB/MLT/CD when compared with BHB/MLT/PVP may be a consequence of increased ability to retain melatonin in solution upon dilution (or administration). PVP forms soluble complexes with aromatic compounds in aqueous solutions, stabilized by non-specific hydrophobic interactions (iceberg theory; (402)). On the other hand, melatonin forms unimolecular inclusion complexes with HPβCD, and the 5-methoxy indole end of melatonin is included within the hydrophobic cavity of HPβCD (402). Addition of water-soluble polymers, such as PVP, improves the stability of the complex formed between cyclodextrin and guest molecules (403, 404). Furthermore, HPβCD was shown to be protective against ischemia/reperfusion injury in rats (405), an effect that may have been enhanced by PVP-induced improvements in circulation (406).

Limitations and future directions

Previous studies established that treatment with 4 M BHB/43 mM MLT/20% DMSO significantly improves survival in multiple preclinical models of hemorrhagic shock (231-233). To test the effects of BHB/MLT treatment on early shock-induced mortality, we utilized a lethal rat model in which untreated hemorrhage resulted in a mean survival

time < 72 minutes. This model did not include an injury or resuscitation component other than BHB/MLT treatment. Further studies are needed to assess whether the beneficial effects of BHB/MLT/PVP and BHB/MLT/CD demonstrated in this pilot study are retained in a more comprehensive shock model.

Conclusions

In this study, we developed novel BHB/MLT formulations which improve survival in a rat hemorrhagic shock model. Our experiments indicate that BHB/MLT/PVP and BHB/MLT/CD constitute promising candidates for the clinical treatment of acute hemorrhagic shock. Optimized lyophilization protocols generated solid dispersions with reconstitution times of less than two (BHB/MLT/PVP) or 3 minutes (BHB/MLT/CD), respectively. Both novel solutions had significantly lower hemolysis-inducing potentials than 4 M BHB/MLT/DMSO, while other markers of toxicity did not differ significantly between treatment formulations. While efficacy of the original solution was retained when DMSO was replaced with PVP, BHB/MLT containing HPβCD, PVP and PEG may be superior to the established formulation containing 20% DMSO.

Chapter 5. D-β-Hydroxybutyrate and Melatonin Mechanism of Action

Introduction

The experiments presented in the previous chapters were concerned with the optimization of BHB/M administration, dose and treatment formulation. Despite the increasing characterization of BHB/M as a treatment for hemorrhagic shock one question remains: How does it work? The objective of the work presented in this chapter was to determine the mechanism of action behind the beneficial effects of BHB/M in an *in vitro* ischemia/reperfusion model. Elucidating the mechanism of action will both yield valuable insights on the BHB/M treatment and help identify targets for future treatment approaches for shock and polytrauma.

Hemorrhagic shock is characterized by decreased tissue perfusion, cellular oxygen and energy supply, resulting in impaired mitochondrial respiration and ATP production (5, 8, 281). Paradoxically, when tissue perfusion is restored during resuscitation, rapid mitochondrial activation results in increased production of reactive oxygen species, oxidative stress, and consequently, cell and tissue damage (5, 8). As described in the introduction, both BHB and melatonin exert beneficial effects in hemorrhagic shock (153, 154, 188, 189, 226, 227, 231-233, 407).

We propose that BHB and melatonin improve outcomes after hemorrhagic shock by influencing cellular energy metabolism and oxidative stress (Figure 5.1). BHB, a ketone body and a physiological energy source, increases mitochondrial function and levels of ATP after ischemia/reperfusion (60, 133, 152, 408). The observation that pigs treated with BHB/M showed increased oxygen consumption during hemorrhagic shock suggests that this effect is exerted even in the context of decreased oxygen supply (232). Defects in the electron transport chain result in production of reactive oxygen species and mitochondrial damage (213). Consequently, BHB-mediated protection of mitochondrial function during suboptimal oxygen supply may diminish ischemia/reperfusion-induced oxidative stress. Indeed, BHB significantly decreased markers of oxidative stress after ischemia/reperfusion (285, 408).

Depletion of cellular antioxidant defense elements during ischemia makes cells particularly vulnerable to oxidative stress (240, 409-412). Consequently, treatment with

melatonin, a radical scavenger, may attenuate reperfusion injury (201, 202). Indeed, countless studies have documented decreased markers of oxidative stress and injury after melatonin treatment in hemorrhagic shock and ischemia/reperfusion (222, 224, 226, 413-415). In addition to acting as a radical scavenger itself, melatonin enhances the activity of endogenous antioxidants and antioxidant enzymes, including glutathione, superoxide dismutase, and glutathione peroxidase (196-199). Furthermore, some of the metabolites generated via the reaction of melatonin with reactive oxygen species exhibit antioxidant activity themselves, thereby potentiating its effect (203-206). By scavenging reactive oxygen species in mitochondria, melatonin protects respiration and mitochondrial function in the context of oxidative damage (215-217, 219, 286, 416). Melatonin treatment increased activities of complexes I and IV of the electron transport chain in the brain and the liver under basal conditions as well as after treatment with the mitochondrial toxin ruthenium red (216). Furthermore, melatonin treatment prevented shock-induced decrease in liver ATP levels in rats subjected to trauma and hemorrhage (226).

Based on the described studies, we proposed the hypothesis that BHB/M increases survival of hemorrhagic shock and trauma by improving mitochondrial function during blood loss and resuscitation (Figure 5.1). We further proposed that increased mitochondrial respiration results in an improved energy balance and decreased oxidative stress, and ultimately decreased ischemia/reperfusion injury in hemorrhagic shock and resuscitation.

To test our hypothesis, we established an *in vitro* ischemia/reperfusion model in H9c2 cardiomyoblasts. We hypothesized that treatment with BHB/M decreases oxidative stress and improves mitochondrial function in this *in vitro* ischemia/reperfusion model.

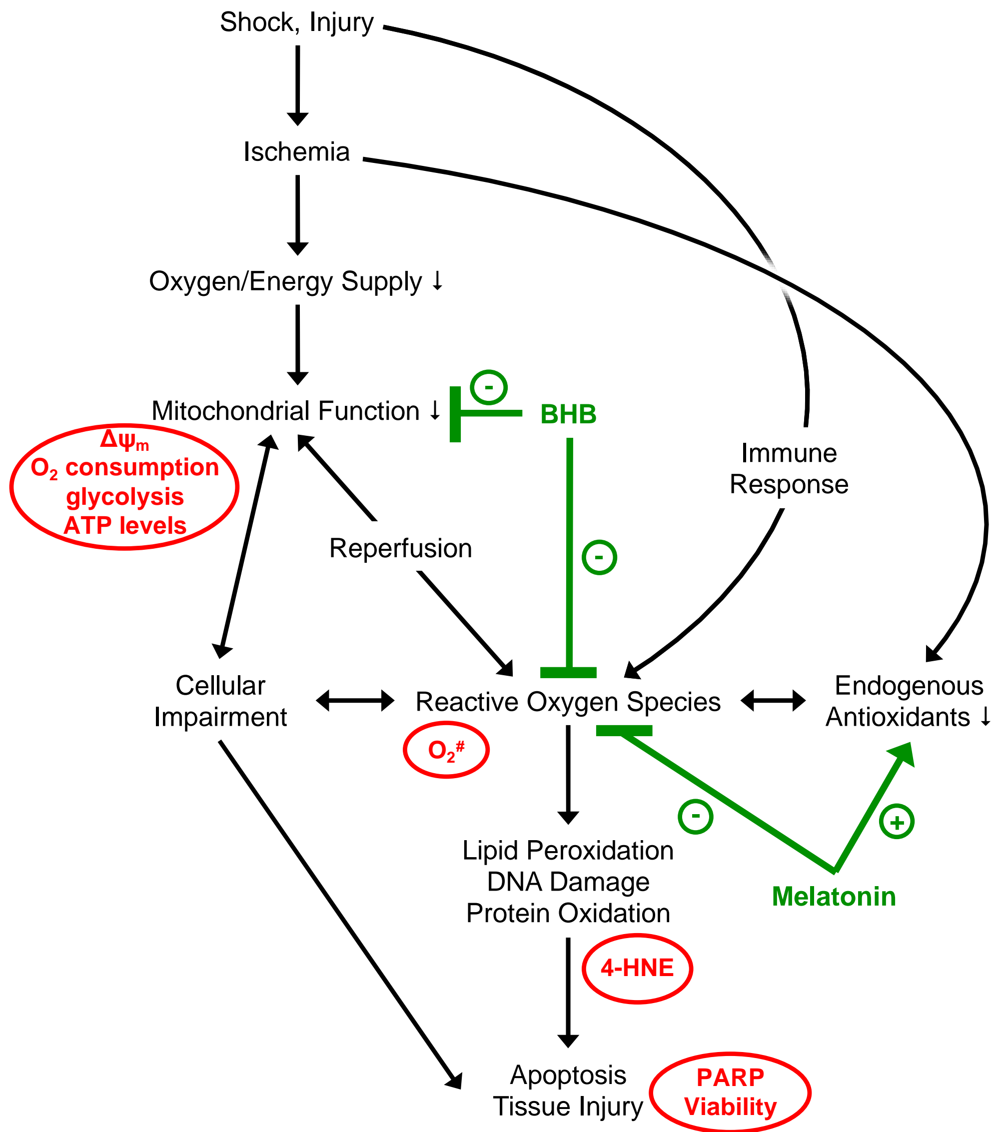


Figure 5.1. Proposed mechanism of action for BHB and M in the treatment of hemorrhagic shock and injury. Limited tissue oxygen supply during severe blood loss and rapid mitochondrial activation upon reperfusion result in oxidative stress, decreased mitochondrial function, and tissue injury. BHB improves mitochondrial respiration, thereby mitigating the induction of oxidative stress during reperfusion. Melatonin prevents oxidative stress-induced cell damage via its direct antioxidant effect and by enhancing endogenous antioxidant systems. Furthermore, melatonin improves mitochondrial function directly, thereby decreasing reperfusion-induced oxidative stress and enhancing the effect of BHB. Red circles depict markers evaluated in our *in vitro* ischemia/ reperfusion model.

Materials and methods

Cell culture and oxygen glucose deprivation

H9c2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in T75 flasks with cell culture medium and maintained at 37ºC in 5% CO_2/95% air and split before reaching confluency. All cell experiments were conducted on plates coated with 5 µg/cm^2 rat tail collagen 1 to avoid cell loss during assays.

Cell culture medium was Dulbecco's modified eagle medium containing 1.5 g/l $NaHCO_3^-$, 5.5 mM glucose, 1 mM Na-pyruvate, 4 mM l-glutamine, 100 I.U./ml penicillin/streptomycin and supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). Oxygen glucose deprivation (OGD) medium was Dulbecco's modified eagle medium containing 1.5 g/l $NaHCO_3^-$, 100 I.U./ml penicillin/streptomycin but no glucose, glutamine, pyruvate or fetal bovine serum. The day before OGD induction, OGD medium was deoxygenized in a modular incubator chamber (Billups-Rothenberg Inc, Del Mar, CA) which was flushed with 5% CO_2/95% N_2 in the morning (20 l/min, 8 min) and again in the evening (20 l/min, 3 min) to remove residual oxygen released from the medium.

Cells were exposed to three hours of oxygen glucose deprivation followed by thirty minutes of reoxygenation (OGD/R). Cells were seeded 24 hours before the beginning of each experiment, and had reached confluency at the beginning of OGD/R induction. On the day of the experiment, cells were washed with OGD medium, then OGD medium and treatment solutions were added. Cells were kept in the modulator incubator chamber, which was flushed with 5% CO_2/95% N_2 immediately (20 l/min, 8 min) and again after one hour (20 l/min, 3 min) and placed in the incubator. After three hours of OGD, to initiate reoxygenation, OGD medium was carefully removed and replaced with cell culture medium containing the respective treatments. Normoxia cells were exposed to the same wash steps as the OGD group, but cell culture medium was used at all steps.

Cell treatment

Cells were treated with 5 mM BHB, 250 µM melatonin, 5mM BHB/250 µM melatonin or vehicle (0.025% DMSO), respectively, during OGD and reoxygenation. Melatonin was dissolved in DMSO to generate a 1 M stock solution. BHB was dissolved in OGD medium and NaOH was added to generate a 1 M stock solution at pH 7.4.

Treatments were prepared as 10x solutions in cell culture or OGD medium, respectively. Treatment solutions were added at the onset of OGD and then again reoxygenation to reach the desired treatment concentrations.

Viability assay

For cell viability assays, cells were grown on six well plates at 520,000 cells/well for 24 hours before induction of OGD/R. OGD medium, reperfusion medium and dissociated cells were collected for each well. The cell suspension was centrifuged for 5 min at 100 g, the supernatant was discarded and cells were resuspended in 500 µl cell culture medium per sample. Viability was determined via trypan blue exclusion assay utilizing a Nexcelom Cellometer ™ Auto T4 (Nexcelom Bioscience, Lawrence, MA).

ATP assay

Cells were seeded on white opaque 96-well plates (17,500 cells/well) for 24 hours before the start of the experiment. After OGD and reoxygenation, the plate was removed from the incubator and placed in the cell culture hood for 30 minutes to cool to room temperature. 100 µl CellTiter-Glo® 2.0 assay solution (Promega, Corporation, Madison, WI) was added per well and the plate was protected from light and put on a shaker for 5 minutes to induce cell lysis. The plate was equilibrated for 10 minutes and luminescence was measured in a BioTek Synergy 2 Reader (BioTek, Winooski, VT).

Mitochondrial membrane potential assay

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed via 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) assay. JC-1 accumulation results in a shift of fluorescence emission from green to red, as monomers emit light at 525 nm, while aggregates emit light at 590 nm. JC-1 accumulates potential-dependently in mitochondria. The ratio of red to green fluorescence is an indicator of mitochondrial polarization, with a decrease in red-to-green ratio indicating mitochondrial depolarization (210).

JC-1 (Thermo Fisher Scientific, Rockford, IL) was dissolved in DSMO to generate a 5mM stock solution, which was aliquoted and frozen at -20ºC until further use. JC-1 was added to cell culture medium with 30 µM verapamil (417) (Sigma-Aldrich, St Louis, MO) to generate a 5 µM JC-1 working solution. The working solution was protected from light, centrifuged for 2,000 g for 5 minutes and used immediately for the assay. Cells were

seeded on black 96-well plates with transparent bottoms (17,500 cells/well) for 24 hours before the beginning of the experiment. After OGD, JC-1 working solution and cell treatments were added. Cells were protected from light and incubated at 37 ºC in 5% CO_2/ 95% air. After 30 minutes, plates were carefully washed twice with Hank's balanced salt solution (HBSS), then 100 µl HBSS were added to each well. Fluorescence of JC-1 monomers (excitation 485±10 nm, emission 528±10 nm) and aggregates (excitation 530±12.5 nm, emission 590±17.5 nm) were quantified using a BioTek Synergy 2 Reader (BioTek, Winooski, VT). The uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 50 µM) was used as a depolarization control.

MitoSOX™ red assay

Mitochondrial production of reactive oxygen species (ROS) was assessed with the MitoSOX™ Red assay. MitoSOX red is a lipophilic, cationic, fluorogenic dye that accumulates preferentially in polarized mitochondria (418). MitoSOX red is oxidized by superoxide anions into to a product that is highly fluorescent after binding to nucleic acid.

MitoSOX ™ (Molecular Probes, Eugene, OR) was dissolved in DMSO to generate a 5 mM stock solution, which was diluted in cell culture medium with 30 µM verapamil to generate a 3 µM working solution, which was used immediately for the assay. Cells were seeded on black 96-well plates with transparent bottoms (17,500 cells/well) for 24 hours before the beginning of the experiment. After OGD and reoxygenation, cell culture medium was carefully removed and MitoSOX working solution was added (100 µl/well). The plate was protected from light and incubated at 37 ºC in 5% CO_2/95% air. After 20 minutes, plates were carefully washed with HBSS, and HBSS 100 µl HBSS were added to each well. The plate was again incubated at 37 ºC in 5 % CO_2/95 % air for twenty minutes. Fluorescence (excitation 485±10 nm, emission 360±20 nm) was quantified using a BioTek Synergy 2 Reader (BioTek, Winooski, VT). Antimycin A (AA, 2.5 µM), a known inducer of mitochondrial ROS, was added as a positive control. After fluorescence measurements, HBSS was carefully removed from each well and 0.5 % crystal violet solution was added (50 µl/well). Cells were protected from light and incubated for 20 minutes while shaking, washed four times in water, and crystal violet was dissolved with methanol (200 µl/well) for 40 minutes on a shaker. Absorbance was quantified at 570 nm using a BioTek Epoch plate reader (BioTek, Winooski, VT).

MitoSOX fluorescence/crystal violet absorbance was normalized to the average fluorescence/absorbance of the OGD/R plate for each experiment.

Seahorse assay

Cellular respiration (oxygen consumption rate, OCR) and proton efflux (extracellular acidification rate, ECAR) were measured using a Seahorse XF or Seahorse XFe analyzer (Agilent Technologies, Santa Clara, CA). Cells were seeded in 24-well XF or XFe cell culture plates (15,000 cells/well) 48 hours before the beginning of the experiment. After OGD and reoxygenation, plates were carefully washed with Seahorse assay medium (unbuffered DMEM supplemented with 10 mM glucose, 2 mM l-glutamine and 1 mM pyruvate, pH 7.4), then 500 µl assay medium was added to each well and the plate was incubated at 37ºC for one hour in a non-CO_2 incubator.

Cells were evaluated at baseline, and then in sequence after addition of oligomycin (1 µM, ATP synthase inhibitor), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 µM, uncoupler) and rotenone/antimycin A (0.5 µM, complex I and III inhibitors, respectively) to evaluate key parameters of mitochondrial function. Each measuring step included mixing of medium (3 min), waiting (2 min) and measuring (3 min). Key parameters of mitochondrial function were calculated using the second of three measurements for each condition. Basal respiration was calculated as (baseline OCR – rotenone/antimycin A OCR), proton leak was calculated as (oligomycin OCR – rotenone/antimycin A OCR), ATP turnover was calculated as (baseline OCR – oligomycin OCR), and maximum respiratory capacity was calculated as (FCCP OCR - rotenone/antimycin A OCR). Glycolytic reserve was calculated as (oligomycin ECAR – baseline ECAR). Optimal cell density and FCCP concentration for untreated cells were established in initial experiments (not shown). After the assay, cells were lysed with 0.1 % SDS overnight and OCR and ECAR values were normalized to protein content. All inhibitors were purchased from Agilent (Agilent Technologies, Santa Clara, CA) and prepared immediately before the experiment.

Western blot

Levels of 4-hydroxynonenal (4-HNE)-modified proteins, cleaved Poly (ADP-ribose) polymerase 1 (PARP-1) and βActin were analyzed by Western Blot. Cells were plated on six-well plates for 24 hours (520,000 cells/well) before exposure to OGD/R. After

OGD/R, cells were dissociated with trypsin-EDTA and washed with HBSS (both Gibco, Grand Island, NY) and centrifuged at 500 g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in RIPA lysis buffer containing 1x cOmplete protease inhibitor cocktail™ (Roche Diagnostics GmbH, Mannheim, Germany). Cells were lysed on an end-over-end rotator for 1 hour at 4ºC and centrifuged for 10 minutes at 8,000 g. The supernatants were collected and stored at -80 ºC until use for western blotting. Samples were quantified with the Pierce™ BCA protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's instructions.

Cell lysates were subjected to sodium dodecyl sulfate-PAGE on a 12% gel. The maximum available amount of protein was loaded for each gel, with equal amount of protein in each lane (10-37 μg/lane). Proteins were transferred to a 0.2 μm polyvinylidene fluoride membrane at 20 V for 7 minutes using the iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, Rockford, IL). Membranes were blocked in 5% skim milk in tris-buffered saline with 0.05 % Tween 20 for 1-3 hours and incubated with primary antibodies at 4 ºC overnight. Primary antibodies were purchased from abcam (anti-4-HNE rabbit polyclonal, 1:250) or Cell Signaling Technologies (anti-βActin mouse monoclonal, 1:1000; anti-PARP-1 rabbit monoclonal, 1:500). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary IgG (1:20,000; Thermo Fisher Scientific, Rockford, IL) for 1 h. Proteins were detected using SuperSignal™ West Pico PLUS Chemoluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) using a LI-COR Odyssey® Fc imager (LI-COR Biotechnology, Lincoln, NE). Densitometric analysis of protein bands was performed with Image Studio ™ Software version 5.2 (LI-COR Biotechnology, Lincoln, NE). Protein bands were normalized to βActin expression and the average density of the untreated normoxia or OGD/R group, respectively.

Statistical analysis

Statistical analysis was carried out using SPSS version 23 for Macintosh (SPSS, Chicago, IL). At least three treatment replicates were tested in each experiment. These replicates were averaged, and averages from experiments were pooled for analysis. Differences between groups were detected by one-way analysis of variance. Levene's test was used to assess homogeneity of variances. When variances were equal, Dunnett multiple comparisons, using the normoxia or OGD/R group as control, were used. When variances were unequal, Games-Howell multiple comparisons were used. Data are

presented as means ± standard deviations. A p-value <0.05 was considered statistically significant.

Results

OGD/R and dosing optimization

We utilized oxygen glucose deprivation followed by reoxygenation (OGD/R) as an *in vitro* ischemia/reperfusion model (419-421). Initial experiments showed that three hours of OGD followed by thirty minutes of reoxygenation induced maximum decreases in cellular ATP levels without significant detachment of H9c2 cells during the assays (not shown). We then conducted dose-response experiments to evaluate the effects of increasing concentrations of BHB and melatonin on cellular ATP levels after OGD/R. Our experiments showed dose-dependent decreases in cellular ATP levels after treatment with BHB and melatonin, suggesting that > 10 mM BHB concentrations and > 1 mM melatonin concentrations impaired cellular health (Figure 5.2).

In our *in vivo* hemorrhagic shock studies, treatment with 4 M BHB/43 mM M resulted in peak serum concentrations of approximately 5-10 mM BHB and 30-70 µM melatonin, respectively (231-233, 293, 371). Treatment with 1 - 10 mM BHB improved survival, decreased apoptosis, increased ATP levels and inhibited ROS production in models of hypoxia, glucose deprivation, and glutamate or mitochondrial toxicity *in vitro* (150, 282, 422-425). Various studies have evaluated the effects of melatonin in H9c2 cells, and concentrations of 10 µM – 1 mM significantly increased viability and decreased apoptosis and oxidative stress after ischemia/reperfusion (426-430). In an *in vitro* trauma model in H9c2 cells, pretreatment with 100 µM melatonin significantly increased viability, decreased apoptosis and attenuated mitochondrial fission (431).

Based on these data, we conducted initial experiments to evaluate the effects of selected combinations of BHB and melatonin on mitochondrial ROS production (Figure 5.2). Although differences were not significant at this initial stage, our comparison indicated that 5 mM BHB/250 µM M effectively inhibited mitochondrial ROS production. Hence, this combination was used in all subsequent experiments.

Effect of BHB and melatonin on cell viability and apoptosis after OGD/R

Cell viability and the number of cells per well did not differ significantly after normoxia or OGD/R with or without treatment with 5 mM BHB, 250µM melatonin, 5 mM BHB/250 µM melatonin or vehicle (Figure 5.3 A, B; not shown). Induction of apoptosis was evaluated by quantification of cleaved PARP-1. Protein levels of cleaved

PARP-1 did not differ significantly between normoxic and OGD/R H9c2 cells, and treatment with BHB/M or vehicle had no significant effect on apoptosis (Figure 5.3 C-E).

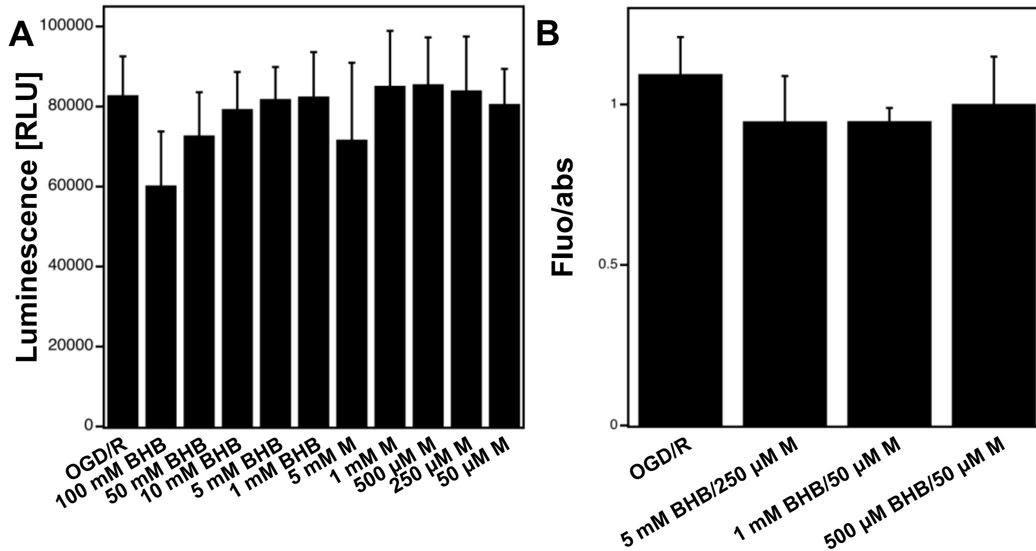


Figure 5.2. Cellular ATP levels and mitochondrial ROS production after OGD/R and treatment with BHB and melatonin. (A) ATP levels in H9c2 cells were measured with the CellTiter-Glo® 2.0 assay after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion. (B) Mitochondrial ROS production in H9c2 cells was measured via MitoSOX™ Red assay after 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 3). Cells were treated during OGD and reperfusion with different concentrations of BHB, M or BHB/M, respectively. * p<0.05 vs OGD/R. RLU – relative luminescence unit

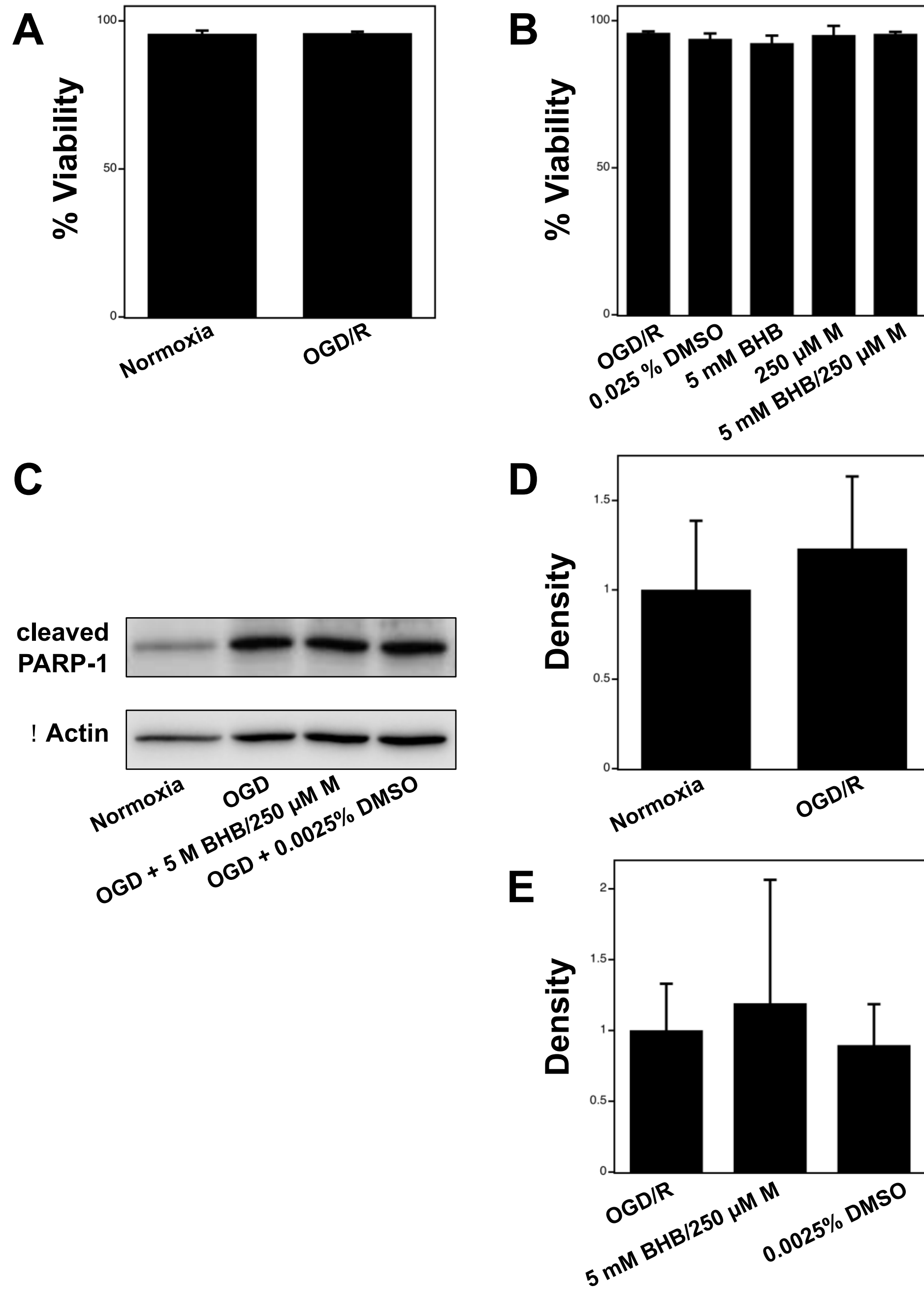


Figure 5.3. (A, B) Cell viability and (C-E), apoptosis after OGD/R and treatment with 5 mM BHB/250 µM M. (A, B) H9c2 cell viability was measured via trypan blue assay after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 3). (C) Cleaved PARP-1, a marker of apoptosis, was detected via Western blot after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion and (D, E) quantified via densitometry analysis (n = 4). Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively.

Cellular ATP levels after OGD/R and treatment with BHB and melatonin

To test the effects of BHB and melatonin on cellular bioenergetics, we evaluated cellular ATP levels in H9c2 cells with the bioluminescent CellTiter-Glo® 2.0 assay. OGD/R significantly decreased ATP levels in H9c2 cells to a level similar to that after treatment with antimycin A, an inhibitor of complex III of the electron transport chain (Figure 5.4). Treatment with 5 mM BHB, 250 µM melatonin, or 5 mM BHB/250 µM melatonin during OGD/R had no effect on cellular ATP levels.

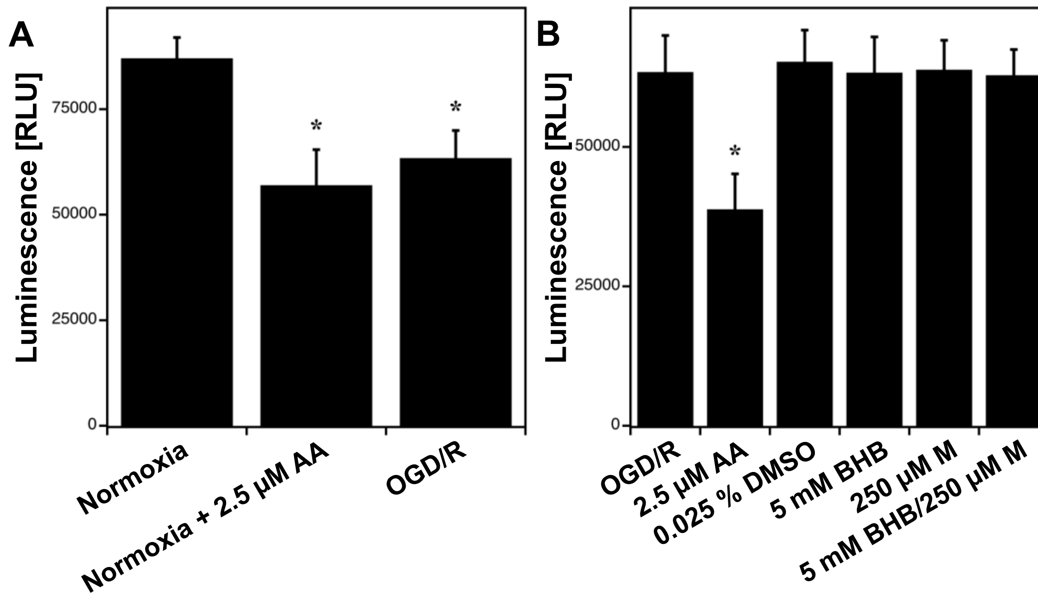


Figure 5.4. Cellular ATP levels after OGD/R and treatment with 5 mM BHB/250 µM M. (A, B) ATP levels in H9c2 cells were measured with the CellTiter-Glo® 2.0 assay after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 3-4). Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively, 2.5 µm AA (positive control) was added during reoxygenation. * $p<0.05$ vs (A) normoxia or (B) OGD/R. RLU – relative luminescence unit

Mitochondrial ROS production and lipid peroxidation after OGD/R and treatment with BHB and melatonin

To test the effects of BHB and melatonin on oxidative stress induction, we evaluated mitochondrial ROS production with the MitoSOX™ Red assay. As intended, treatment with antimycin A, an inducer of mitochondrial ROS, significantly increased MitoSOX Red fluorescence in both normoxic and OGD/R cells (Figure 5.5 A, B). Mitochondria-derived ROS-levels were significantly increased after OGD/R when compared to normoxic cells. However, treatment with BHB and melatonin did not significantly alter mitochondrial ROS production. Overproduction of mitochondrial ROS causes oxidation of polyunsaturated fatty acids in cellular membranes (lipid peroxidation). The mitochondrial membrane contains high levels of cardiolipin and other unsaturated fatty acids, making it especially susceptible to lipid peroxidation (432). Lipid peroxidation results in the disruption of mitochondrial functions, including the electron transport chain (433). To evaluate the effects of OGD/R and BHB and melatonin treatment on lipid peroxidation, we quantified 4-hydroxynonenal (4-HNE) protein adducts via Western blotting. There were no significant changes in 4-HNE protein adducts after OGD/R with or without treatment with BHB/M or vehicle (Figure 5.5 C-D).

Mitochondrial membrane potential after OGD/R and treatment with BHB and melatonin

Mitochondrial membrane potential was evaluated with the fluorescent dye JC-1. JC-1 accumulates potential-dependently in mitochondria, resulting in a fluorescence shift from green to red. A decrease in red-to-green ratio thus indicates a decrease in mitochondrial membrane potential (210). As expected, treatment with the mitochondrial uncoupler CCCP significantly decreased mitochondrial membrane potential in both normoxic and OGD/R cells (Figure 5.6). OGD/R did not result in changes in mitochondrial membrane potential when compared to normoxic cells. However, treatment with a combination of 5 mM BHB/250 μM melatonin during OGD/R significantly decreased mitochondrial membrane potential when compared to untreated cells.

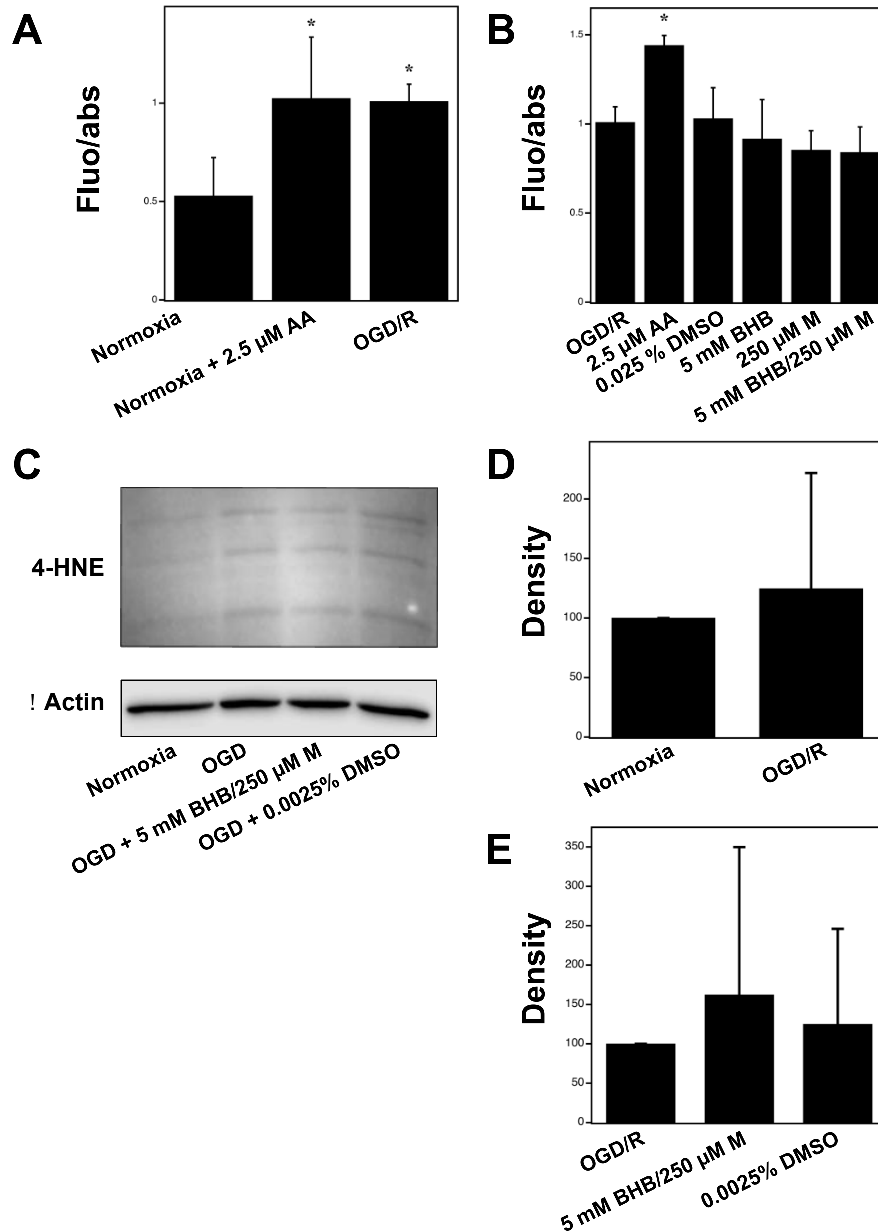


Figure 5.5. (A, B) Mitochondrial ROS production and (B-E) lipid peroxidation after OGD/R and treatment with 5 mM BHB/250 µM M. (A, B) Mitochondrial ROS production was measured with the MitoSOX™ Red assay after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n =5-8). (C) 4-HNE protein adducts were detected via Western blot after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion and (D, E) quantified via densitometry analysis (n = 5). Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively, 2.5 µM AA (positive control) was added after the HBSS wash step. * $p<0.05$ vs (A) normoxia or (B) OGD/R.

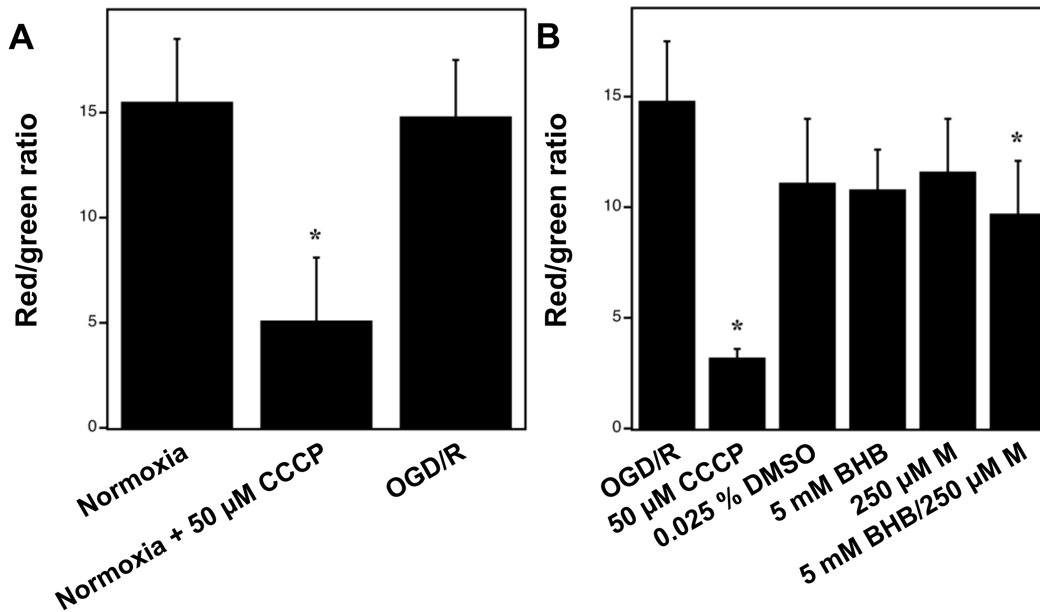


Figure 5.6. (A, B) Mitochondrial membrane potential after OGD/R and treatment with 5 mM BHB/250 µM M. A, B) mitochondrial membrane potential was quantified using the JC-1 assay after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 4-5). A decrease in red/green ration indicates a depolarization of mitochondrial membrane potential. Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively, 50 µm CCCP (positive control) was added during reoxygenation. * $p<0.05$ vs (A) normoxia or (B) OGD/R.

Mitochondrial respiration after OGD/R and treatment with BHB and melatonin

Mitochondrial respiration and glycolysis were analyzed with the Seahorse extracellular flux analyzer. Oxygen consumption of H9c2 cells was measured in the basal state and then in sequence after addition of oligomycin (to inhibit ATP synthase), FCCP (to depolarize the inner mitochondrial membrane potential and induce maximal respiration) and antimycin A/rotenone (to inhibit oxygen utilization by the electron transport chain) and key respiratory markers were calculated. OGD/R or treatment with BHB and melatonin during OGD/R did not significantly change basal and maximal respiration, proton leak or ATP turnover (Figure 5.7, Figure 5.8). The extracellular acidification rate (ECAR) is predominantly driven by cellular excretion of lactic acid, and is therefore a marker of glycolysis (434). Although the differences were not significant, OGD/R cells appeared to exhibit increased glycolysis when compared to normoxia cells. Furthermore, neither the basal ECAR nor the glycolytic reserve differed significantly between BHB/M-treated and control OGD/R cells.

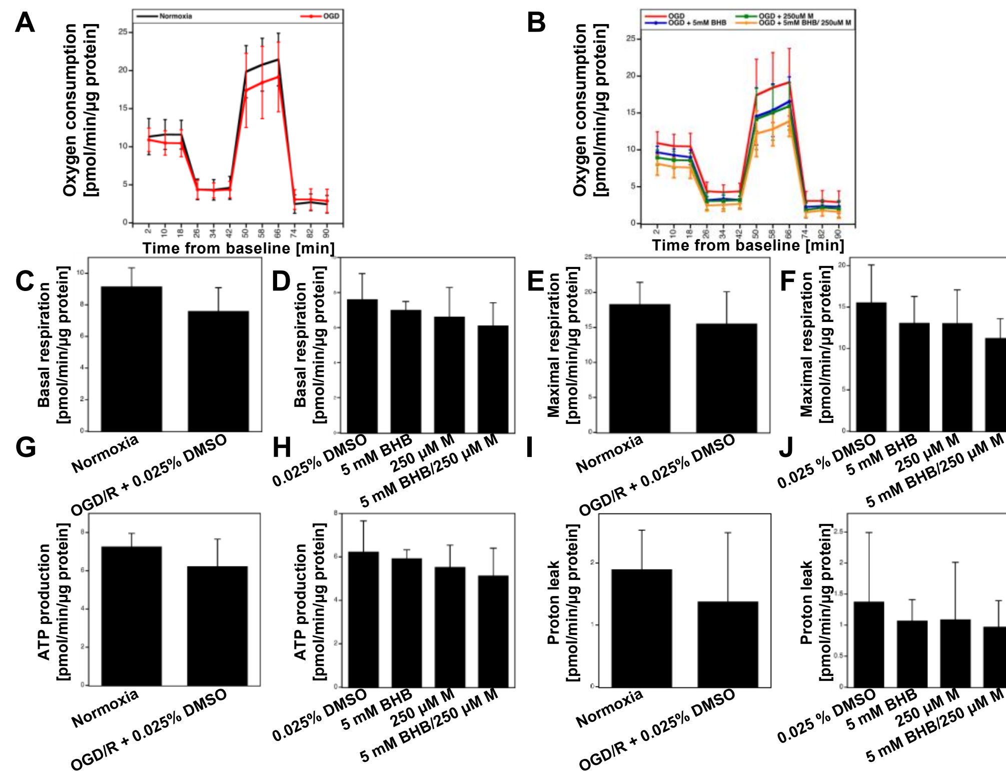


Figure 5.7. Mitochondrial respiration after OGD/R and treatment with 5 mM BHB/250 µM M. (A, B) Oxygen consumption over time was determined in a Seahorse XFe extracellular flux analyzer after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 5-8). Oxygen consumption values were used to calculate (C, D) basal respiration, (E, F) maximal respiration, (G, H) ATP production and (I, J) proton leak as described in the methods section. Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively.

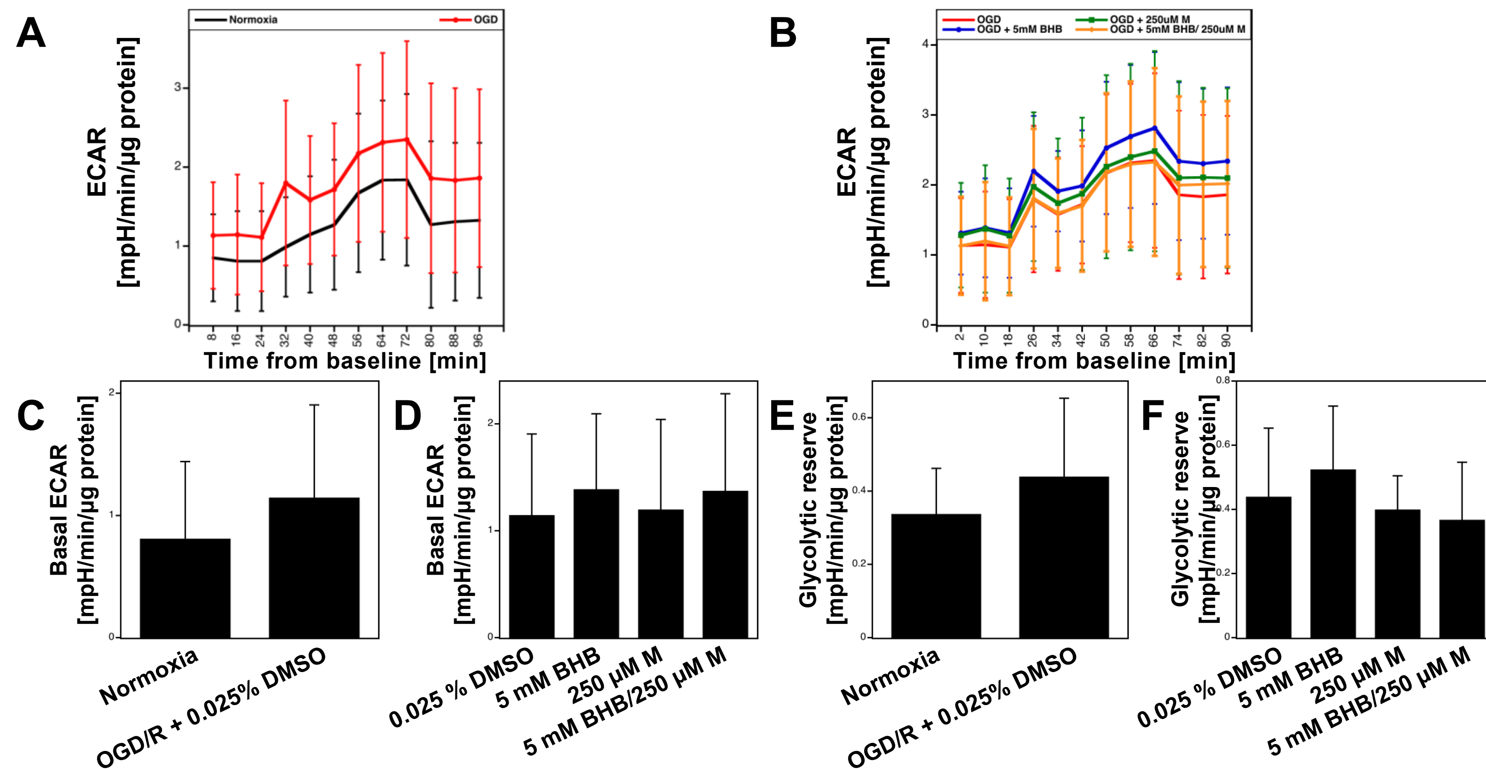


Figure 5.8. Glycolysis after OGD/R and treatment with 5 mM BHB/250 µM M. (A, B) glycolysis over time was determined via extracellular acidification rate (ECAR) in a Seahorse XFe extracellular flux analyzer after exposure to 3.5 hours of normoxia or 3 hours of OGD followed by thirty minutes of reperfusion (n = 6-8). ECAR values were used to calculate (C, D) basal glycolysis and (E, F) glycolytic reserve as described in the methods section. Cells were treated during OGD and reperfusion with 0.025% DMSO (vehicle), 5 mM BHB, 250 µM M or 5 mM BHB/250 µM M, respectively.

Discussion

Here, we evaluated the effects of BHB and melatonin treatment in H9c2 cells exposed to three hours of OGD followed by thirty minutes of reoxygenation. We hypothesized that treatment with BHB/M would decrease oxidative stress and improve mitochondrial function in this *in vitro* ischemia/reperfusion model. We used H9c2 immortalized cardiomyoblasts, as these cells exhibit high ATP levels, mitochondrial mass and respiratory activity, and are commonly used in ischemia/reperfusion models (435-439). The heart is one of the organs with the most active ketone body metabolism, underlining the importance of these energy substrates for cardiac cells (133, 440). Ischemia is characterized by decreased oxygen supply, increased CO_2 concentrations, limited nutrient availability and impairment of waste removal with increased acidity (421, 441, 442). To model ischemia/reperfusion, we exposed H9c2 cells to three hours of oxygen glucose deprivation, followed by thirty minutes of reoxygenation. To our surprise, treatment with 5 mM BHB/250 µM M during OGD/R decreased mitochondrial membrane potential, but had no significant effects on cellular ATP levels, mitochondrial respiration or ROS production when compared to untreated controls.

Mitochondrial membrane potential ($\Delta\psi_m$) is an indicator of cellular health, and it plays a significant role in mitochondrial Ca^{2+} homeostasis, ROS production (210, 443, 444) and apoptosis (445). Electron transfer through the electron transport chain fuels the extrusion of protons from the mitochondrial matrix into the cytosol, creating an electrochemical gradient. The energy stored in this gradient, the proton motive force, the driving force for ATP production through complex V (ATPase) during oxidative phosphorylation. $\Delta\psi_m$ makes up the majority of the proton motive force, the proton gradient is the second contributor (209, 210).

Dependent on the magnitude, decreases in $\Delta\psi_m$ can be beneficial or detrimental (446, 447). Ischemia/reperfusion and other insults can lead to opening of the mitochondrial permeability transition pore (mPTP) (448). While transient mPTP opening has been detected without detrimental effects (449), irreversible mPTP opening is associated with collapse of the $\Delta\psi_m$, impaired mitochondrial respiration, ATP production, cell swelling, apoptosis and cell death (450, 451). In contrast to $\Delta\psi_m$ collapse, slight decreases in $\Delta\psi_m$ and proton motive force can have beneficial effects. Proton leak, also called uncoupling, is the loss of protons from the mitochondrial matrix independent of ATPase (446). Proton

leak is mediated by uncoupling proteins (UCPs) and Adenine nucleotide translocase (ANT) (452). In a negative feedback loop, mitochondrial ROS and 4-HNE can activate UCP- and ANT-mediated uncoupling, which then decreases ROS production and mitochondrial damage (453-456).

Studies consistently report lowered $\Delta\psi_m$ after hemorrhagic shock and ischemia/reperfusion. However, the nature and consequences of these decreases vary. On the one hand, hemorrhagic shock and I/R injury are associated with mPTP opening, resulting in impaired mitochondrial respiration and lowered ATP production along with increased apoptosis and mild mitochondrial ROS production (86, 125, 457-460). On the other hand, studies have shown that elevated proton leak is protective against ischemia/reperfusion injury and hemorrhagic shock (461-463). The described variations in $\Delta\psi_m$ likely result from model-dependent differences in ischemia/reperfusion severity. In our study, although a decrease in $\Delta\psi_m$ without significant changes in ATP levels or mitochondrial respiration suggests that BHB/M treatment induced proton leak rather than irreversible mPTP opening, there was an nonsignificant trend towards decreased proton leak in BHB/M treated cells (Figure 5.7).

The decrease in $\Delta\psi_m$ in our study is likely the result of the combined effects of BHB, melatonin and DMSO, all of which were previously shown to affect mitochondrial membrane potential (Figure 5.6). In rats, low-dose DMSO treatment reversibly uncoupled respiration in liver mitochondria (464). While DMSO itself did not induce proton leak *in vitro*, the DMSO metabolite dimethyl sulfide exerted an uncoupling effect (464). Melatonin increased UCP gene and protein expression *in vitro* and *in vivo* (465, 466). Melatonin-induced proton leak was associated with increased activity of complex I, III and IV of the electron transport chain, increased ATP levels and reduced ROS production in mouse liver mitochondria (217). BHB preserved $\Delta\psi_m$ after exposure to hypoxia or the mitochondrial toxin rotenone in cultured cells (150, 467), and it increased the threshold for calcium-induced mPTP opening in isolated neocortical mitochondria (468). BHB treatment did not induce uncoupling in mouse dopaminergic neurons (282), however, ketogenic and ketone ester diets, which increase systemic levels of BHB, increase UCP and decrease mitochondrial ROS production (469-471). Hence, BHB is protective against mitochondrial injury and increased BHB levels may induce proton leak.

Decreases in $\Delta\psi_m$ after treatment with BHB/M were not associated with changes in mitochondrial ROS production, cellular ATP level or mitochondrial respiration (Figure 5.4, Figure 5.5, Figure 5.7). This contrasts previous studies in which treatment with 10 μM – 1000 μM melatonin significantly improved viability, preserved cellular ATP levels and attenuated apoptosis and oxidative stress in H9c2 ischemia/reperfusion models (426-431). Treatment with BHB and fasting-induced ketosis increased ATP levels and decreased oxidative stress, organ injury, apoptosis and cell death after ischemia/reperfusion *in vivo* (60, 152, 285, 472, 473). 3 mM BHB decreased cellular apoptosis but did not affect ATP levels in primary neurons exposed to 4 hours of OGD (472). However, treatment with 1 - 10 mM BHB improved survival, decreased apoptosis, increased ATP levels and inhibited ROS production in models of hypoxia, glucose deprivation, and glutamate and mitochondrial toxicity *in vitro* (150, 282, 422-425). The lack of significant treatment effects in our study may be explained by variations in ischemia/reperfusion and treatment regimens utilized. For example, many of the described studies administered melatonin as a pretreatment for multiple hours before ischemia induction, while we administered BHB/M acutely during OGD/R (427-431). Furthermore, while OGD/R significantly decreased cellular ATP levels and increased mitochondrial ROS production in our model, cellular viability, $\Delta\psi_m$ and mitochondrial respiration were unchanged. This indicates that OGD/R did not induce mPTP opening or irreversible mitochondrial damage. Indeed, in our initial optimization experiments, ATP levels in cells exposed to 3-hour OGD recovered after 60 minutes of reperfusion, indicating that ATP decreases were reversible (not shown). With only transient mitochondrial impairment, acute effects of OGD/R and treatment with BHB and melatonin may have been missed in our assays. One might speculate that BHB/M treatment may have exerted more significant effects if increased doses or prolonged treatment had been administered in a more severe OGD/R model. However, this remains to be tested.

We initially hypothesized that treatment with BHB/M would decrease oxidative stress and improve mitochondrial function in our *in vitro* ischemia/reperfusion model. However, our experiments indicate that it is unlikely that the beneficial effects of BHB/M in hemorrhagic shock are mediated through the improvement of mitochondrial function. If mitochondria are not the main target of BHB/M, how does this treatment improve survival after hemorrhagic shock and injury?

BHB/M attenuates shock-induced microvascular endothelial glycocalyx degradation, which is increasingly recognized as a valuable target for hemorrhagic shock resuscitation (103). Furthermore, BHB and melatonin exert immune-modulatory and receptor-mediated effects. Resuscitation with Ringer's solution in which lactate was replaced with an equimolar amount of BHB significantly decreased systemic and local TNFα levels in rat and porcine hemorrhagic shock models (154, 352), and melatonin treatment decreased post-shock levels of inflammatory mediators in rodents (188-192, 226).

BHB-receptor binding has been associated with reduces in energy expenditure via GPR41 antagonism (234) and neuroprotective effects in stroke via hydroxylcarboxylic acid receptor 2 (235), respectively. However, receptor-mediated BHB effects in hemorrhagic shock remain to be determined. Two G-protein-coupled melatonin membrane receptors have been identified in mammals, MT1 and MT2 (174, 175), which are expressed throughout the body in virtually every organ (176). Melatonin also binds to the orphan nuclear hormone receptor family RZR/ROR, which may relay some of its immune-modulatory effects (177). While MT1 and MT2 have been implicated in melatonin antioxidant functions during hemorrhagic shock, the effects of RZR/ROR binding have not yet been tested (207).

Lastly, hemorrhagic shock induces an imbalance in the activity of histone acetylases (HATs) and histone deacetylases (HDACs), resulting in distinct changes in gene expression patterns (236, 237). HDAC inhibition increased survival in rat and swine models of hemorrhagic shock (238). Recent studies have shown that BHB inhibits class 1 HDAC *in vitro* and *in vivo* (239), suggesting that it may exert its beneficial effects through the upregulation of pro-survival pathways. Interestingly, BHB-induced changes in gene expression and metabolic state are closely linked. On the one hand, BHB metabolism provides NADH and $FADH_2$, reducing equivalents for the electron transport chain. On the other hand, changes in NADH and Acetyl-CoA, an intermediate of BHB metabolism, affect histone acetylation (239).

The goal of the work presented in this chapter was to unravel the mechanism behind the beneficial effects of BHB/M treatment in hemorrhagic shock and injury. To our surprise, treatment with BHB and melatonin did not significantly improve markers of mitochondrial function in our *in vitro* ischemia/reperfusion model. Further studies will have to be conducted to unravel the actions of BHB/M in hemorrhagic shock. BHB/M

may exert mitochondrial effects in modified ischemia/reperfusion protocols, or its effects may be a result of glycocalyx protection, receptor binding, immune-modulation, HDAC inhibition alone or in combination.

Chapter 6. Conclusions, Discussion and Future Directions

The research presented in this dissertation is concerned with the characterization and optimization of BHB/M, a treatment for hemorrhagic shock. The preceding chapters describe experiments to evaluate the safety, optimize the dosing, administration and formulation, and to unravel the mechanism of action behind this promising treatment.

BHB/M treatment optimization

Chapters 2-4 are concerned with the optimization of the BHB/M treatment solution and regimen. In our first set of experiments, we evaluated the safety of intravenous and intraosseous 4 M BHB/43 mM M in a porcine model of hemorrhagic shock and polytrauma (Chapter 2). While neither route of administration induced significant changes in organ function or histology, intraosseous infusion of 4 M BHB/43 mM M was associated with mortality exceeding that in the control group. This was likely due to decreased drug serum concentrations, which were observed after intraosseous infusion of 4 M BHB/43 mM Me in both injured and healthy animals.

Our next objective was to define the maximum tolerated dose of 4 M BHB/43 mM M in porcine hemorrhagic shock with polytrauma (Chapter 3a). In this study, all pigs treated with 4x 4 M BHB/43 mM M died within 17 hours after shock, and mortality was higher in pigs receiving 2x 4 M BHB/43 mM M than in those treated with 1x 4 M BHB/43 mM M. Death was likely a result of the interplay of hemorrhagic shock, injury, and BHB/M-induced hypernatremia and increased intraabdominal pressure due to fluid translocation into the interstitial space. Adverse effects were reversible in some of the 2x 4 M BHB/43 mM M treated pigs, but not when 4x 4 M BHB/43 mM M was given. We concluded that in our porcine model of hemorrhagic shock, injury and resuscitation, 2x 4 M BHB/43 mM M is the maximum tolerated dose.

In rats, lowering the BHB concentration in combination with 43 mM M resulted in increased mortality, while survival times were retained when melatonin levels were lowered (233). The goal of our third set of experiments (Chapter 3b) was to test the efficacy of decreased melatonin concentrations in combination with 4 M BHB in porcine hemorrhagic shock with polytrauma. To our surprise, mortality in pigs receiving BHB/M

containing below-standard melatonin concentrations exceeded that in the control group. Our data suggests that the increase death rate was a result of increased post-shock lung and organ injury.

BHB/M has a narrow therapeutic window

In our current model, decreased drug serum concentrations after intraosseous infusion, or treatment with 4 M BHB in combination with decreased melatonin concentrations were associated not just with decreased efficacy but with mortality that exceeded that in the control group. Increasing the administered volume of 4 M BHB/43 mM M was also associated with increased mortality. These studies underline the importance of reaching adequate systemic BHB and melatonin levels and indicate that, at least in the current formulation, BHB/M has a low narrow therapeutic window. What causes this?

As described in chapter 3a, hypernatremia and fluid loss into the extravascular space are the likely culprits behind adverse effects of increased BHB/M volumes. While the studies described in chapters 2 and 3b detected differing severities of organ injury, commonalities between outcomes provide clues on a common mechanism behind increased mortalities in these experiments.

Decreased drug serum concentrations are associated with increased mortality

Decreased drug serum levels associated with increased mortality in two sets of experiments (Chapters 2 and 3b) suggest dose-dependent treatment effects. Indeed, melatonin effects in hemorrhagic shock can be dose-dependent, and systemic or local melatonin concentrations may need to overcome a threshold level to exert beneficial effects. Infusion of 50 mg/kg, but not of 10 mg/kg melatonin prior to resuscitation resulted in lowered resuscitation fluid requirements and decreased serum levels of the inflammatory marker IL-6 in mouse hemorrhagic shock (474). Furthermore, ketone bodies, while generally regarded as anti-inflammatory, may increase inflammation at high doses (188, 359-361). With systemic melatonin levels insufficient to counteract the effects of shock and injury, BHB may have exacerbated trauma-induced inflammation.

All groups experienced hypernatremia

Another commonality between studies is hypernatremia, which was observed in all BHB/M treated pigs at varying severities. The adverse effects of hypernatremia have been described in chapter 3a. Severe hypernatremia can lead to cell shrinking and subsequent rupture of meningeal vessels, resulting in subarachnoid hemorrhage (302, 309-312). Retrospective review of the necropsy reports produced for pigs treated with LR or 1x 4 M BHB/43 mM M showed that cerebral or spinal cord hemorrhage was reported in 6 of 14 BHB/M IV, 4 of 12 BHB/M IO, but only 1 of 16 LR animals. This data was not systematically acquired for all animals, and was therefore not further analyzed or included in the original publication. While is it impossible to say what caused the hemorrhage, we cannot rule out that this was a consequence of hypernatremia. However, it should be noted that hypernatremia was transient, and that uninjured pigs receiving up to 2x 4 M BHB/43 mM M remained in good health despite mild-to-moderate hypernatremia (Chapter 2). This indicates that mild-to moderate hypernatremia alone was not lethal in pigs, but likely exacerbated trauma-associated adverse effects. Alternatively, hemorrhage may be associated with a shock-induced coagulopathy (5, 7). In our studies, surviving pigs did not exert differences in Glasgow coma scores after extubation. However, we did not assess subtler differences in cognitive functions, and hypernatremia-induced brain damage could have been missed. It is possible that hypernatremia-induced adverse effects exacerbated outcomes when drug serum concentrations were below effective levels. Consequently, treatment toxicity may be decreased by limiting the amount of sodium in the formulation by utilization of ketone esters or alternative BHB salts.

Dose-dependent effects in rat and porcine models

Treatment efficacy of low-dose melatonin BHB/M solutions differed between previously published experiments in rats and our porcine model. In rats, decreasing melatonin in combination with 4 M BHB could be lowered by factor 10^6 without loss of efficacy (233). This discrepancy may be explained by variations in shock models and or physiological differences between species.

First, experiments in rats were conducted in a model of hemorrhagic shock only (231, 233). However, hemorrhagic shock is not an isolated event – most times, severe blood

loss is a consequence of traumatic injury. To mimic the effects of trauma in addition to hemorrhagic shock, we utilized a porcine model in which severe blood loss was combined with pulmonary contusion and liver crush injury (Figure 2.1). As described in the introduction, the mechanic insult of trauma exacerbates the inflammatory response to shock (20-22). Second, rats and pig exhibit differences in immune function and inflammation. Differences include differences in sensitivity to lipopolysaccharide, toll-like receptor expression, and the mononuclear phagocyte system (475, 476). To avoid the variable effects of autotransfusion in pigs, we opted to perform a splenectomy prior to hemorrhage a common procedure in porcine hemorrhagic shock models (e.g. (477-479)). However, splenectomy can impact coagulopathy and inflammation after trauma (480), and may have contributed to observed the differences in outcomes. As we did not evaluate markers of immune response and inflammation, it remains to be tested whether polytrauma, splenectomy or species-induced differences in immune response were a deciding factor.

Melatonin is anti-inflammatory in hemorrhagic shock, and exacerbated inflammation after shock and injury may require increased melatonin doses (188). Hence, melatonin levels that increase survival in rat hemorrhagic shock may not have been adequate in our porcine model.

Lastly, rats and pigs exhibit differences in ketone metabolism. For example, the pig brain has comparably low levels of acetoacetyl-CoA transferase, and pigs exhibit low rates of ketogenesis and fatty acid oxidation when compared to rats (481-483).

BHB/M solutions void of DMSO are effective in hemorrhagic shock

An important step in the preclinical development of BHB/M is the optimization of the treatment formulation. We generated and evaluated two novel BHB/M formulations, BHB/M/PVP and BHB/M/CD, which were freeze-dried to overcome melatonin solution state instability (Chapter 4). BHB/M lyophiles were readily reconstitutable, and the novel formulations had significantly lower hemolysis-inducing potentials than 4 M BHB/MLT/DMSO. In rat hemorrhagic shock, treatment efficacy was retained with the BHB/MLT/PVP formulation, and BHB/MLT/CD may be superior to the original formulation containing 20% DMSO. However, whether treatment efficacy is retained in a more comprehensive model (shock/injury/resuscitation) has not yet been determined.

BHB/M mechanism of action

The objective of our last set of experiments was to unravel the mechanism of action behind BHB/M in an *in vitro* ischemia/reperfusion model (chapter 5). We hypothesized that BHB/M increases survival of hemorrhagic shock and trauma by improving mitochondrial function during blood loss and resuscitation (Figure 5.1). However, treatment with BHB/M did not induce significant differences in ATP levels, mitochondrial ROS production or respiration in H9c2 cells exposed to 3 hours of oxygen glucose deprivation and thirty minutes of reoxygenation. What explains these findings?

BHB/M effects are not mediated through mitochondria or oxidative stress

BHB and melatonin exert various effects that are independent of mitochondria. BHB and melatonin are anti-inflammatory (154, 188-190, 351, 352), and both compounds exert receptor mediated effects (174, 175, 177, 207, 234, 235). BHB inhibits class 1 HDAC, suggesting that it may exert its beneficial effects through the upregulation of pro-survival pathways (239). Lastly, BHB/M attenuates shock-induced microvascular endothelial glycocalyx degradation, which is increasingly recognized as a valuable target for hemorrhagic shock resuscitation (103). BHB/M may be beneficial not through modification of mitochondria and oxidative stress, but through one or more of the above mentioned effects.

The utilized model was not suitable to detect BHB/M induced effects in shock

As discussed in chapter 5, drug dose, treatment length or ischemia severity may have been unsuited to detect significant treatment effects, and modification of these parameters may lead to more pronounced results.

Another potential explanation is that the cell line used in our experiments (H9c2 cells) was not optimal to detect the effects of BHB/M in ischemia/reperfusion injury. H9c2 cells are commonly used in ischemia/reperfusion models, and various studies have detected a role of mitochondrial dysfunction in ischemia/reperfusion injury in this cell line (419, 420, 435, 437-439). The heart has one of the highest metabolic rates in the body (484), and both cardiomyocytes and H9c2 cells contain a large density of mitochondria (436, 485). However, the heart is spared during less serve hemorrhagic shock and consequently might not be the main target of BHB/M treatment (33).

Furthermore, to our knowledge, ketone metabolism has not been evaluated in this cell line, and diminished ketone body metabolism may have reduced the effects of BHB.

Lastly, hemorrhagic shock and trauma do not just affect a single, isolated organ, but invoke a systemic response. Metabolic, inflammatory and other effects are elicited through the interplay of various organs. While melatonin receptors are found throughout the body (176), BHB metabolism is compartmentalized. The liver is the main ketone body producer, yet it does not metabolize ketone bodies itself (142, 149). The heart, brain and kidney are the main utilizers of ketone bodies but produce no or little amounts of ketone bodies (47, 130-132, 143, 144). Utilizing a single cell line makes it impossible to evaluate the interplay between shock-response and metabolism, and an *in vivo* model may be better suited to unravel the mechanism of action behind BHB/M.

Future directions

My thesis research constitutes significant progress in the characterization of BHB/M as a treatment for hemorrhagic shock. However, some important questions remain to be answered.

Is BHB/M effective if treatment onset is delayed?

Our model mimics a situation where treatment is available early after shock, but specialized treatment is delayed. While this scenario is relevant for military and urban trauma, resuscitation cannot always be delivered within minutes of blood loss.

Will the therapeutic window increase if sodium butyrate is replaced with a non-sodium salt?

Our results indicate that hypernatremia is a driving factor of BHB/M-associated adverse effects. If this is the case, administering BHB in the form of non-sodium salts or ketone esters will likely decrease treatment toxicity, thereby widening the therapeutic window of the treatment.

Can an optimized resuscitation protocol increase the therapeutic window for BHB/M? Is BHB/M effective when administered as a less hypertonic solution?

A discussed in chapter 3a, optimization of the resuscitation protocol may improve outcomes after treatment with BHB/M. Administering BHB/M in a less hypertonic solution may diminish adverse effects associated with hypertonicity (chapter 3a). However, giving BHB/M in a less concentrated solution would be associated with a decreased dose or an increased infusion volume. Furthermore, in a pilot study rats, treatment with an isotonic solution containing 140 mM BHB/4.3 mM M or 140 mM BHB/$1.5x10^{-6}$ mM M at one (three-hour shock) or three (one-hour shock) times the shed blood volume resulted in lower survival than in the control group receiving lactated Ringer's solution (486). However, isotonic solutions have not been evaluated in the porcine model.

Are novel BHB/M formulations effective in large animal hemorrhagic shock models?

Non-DMSO formulation of BHB/M were effective in rat hemorrhagic shock. These formulations were easily reconstituted and had a lower hemolytic potential than BHB/M/DMSO. It remains to be tested whether BHB/M/PVP and BHB/M/CD retain efficacy in porcine model in which hemorrhagic shock is combined with polytrauma and resuscitation. Furthermore, the stability of the lyophiles will have to be confirmed.

What is the mechanism of action behind BHB/M?

The experiments described in chapter 5 did not identify the drivers behind BHB/M beneficial effects. Future studies should utilize an *in vivo* approach to examine the effects of BHB/M treatment on inflammation, mitochondrial function, oxidative stress and gene expression after hemorrhagic shock.

Conclusions

My thesis work is concerned with the characterization and optimization of D-beta-hydroxybutyrate and melatonin, a promising treatment for hemorrhagic shock. I have shown that intravenous infusion is the preferred route of administration for BHB/M, and that BHB/M is most effective when administered as a 4 M BHB/43 mM M solution. In the current formulation, BHB/M has a narrow therapeutic window, however, this may be extended by optimization of the treatment formulation or regimen. BHB/M formulations

void of DMSO are effective, and may increase drug stability and decrease adverse treatment effects associated with DMSO. Our experiments suggest that the beneficial effects of BHB/M are not mediated by changes in mitochondrial function. My work highlights the various treatment aspects that must be optimized during preclinical drug development. This research completes multiple essential steps in the translation of BHB/M towards a clinical treatment. It could have important implications for human health, as BHB/M may soon help prevent some of the 5 million injury-related deaths yearly worldwide.

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