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14. ABSTRACT

The primary goal of the research described in the initial Statement of Work is to advance the development of BHB/M so that it is ready for deployment as a portable fast-acting therapy for blood loss. To facilitate deployment, studies will optimize the formulation by increasing our understanding of its mechanism of action in animal models under conditions of 60% blood loss. The low concentration end of the full-factorial design described in Specific Aim 1 was explored in rats. Survival curves were compared after 24 hours using a Wilcoxon test. There was a significant difference ($p<0.05$) in survival: treatment no. 1 (0.4 M D-BHB, 4.3 mM melatonin, 10% DMSO) showed 33% survival; treatment no. 2 (2 M D-BHB, 4.3 mM melatonin, 10% DMSO), 71% survival; treatment no. 3 (4 M D-BHB, 4.3 mM melatonin, 10% DMSO), 78% survival; and treatment no. 18 (4 M D-BHB, 43 mM melatonin, 20% DMSO), 80% survival. Results showed that treatments no. 3 and no. 18 prolong short-term (3 day) survival compared to treatment no.1. From our data and that of Klein *et al* [1], it appears that the animal requires a high concentration of D-BHB in order to overcome the negative effects of hemorrhagic shock.

15. SUBJECT TERMS
hemorrhagic shock, blood loss therapy, D-beta-hydroxybutyrate, melatonin

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Introduction

The Statement of Work submitted to and funded by the US Army Medical Research and Materiel Command in May 2011 outlined three Specific Aims: 1) Perform a dose ranging study of BHB/M components - D-stereoisomer of beta-hydroxybutyrate (D-BHB), melatonin, and dimethyl sulfoxide (DMSO) in hemorrhagically shocked rats, 2) Determine if outcomes from hemorrhagic shock in rats and pigs can be improved by combining BHB/M with the proven hypothermia-promoting adjunct 3-iodothyronamine (T1AM), and 3) Determine the feasibility and benefit of administering a larger volume of a lower molarity BHB/M to hemorrhagically shocked rats. In the same document, it was outlined that the first and second years of funding would be used to complete Specific Aim 1. Hence, the following report is only on experiments conducted towards the completion of our first Specific Aim.

Body

Preliminary Activities

Prior to obtaining funding from the US Army Medical Research and Materiel Command, training of a new staff member was necessary because the previous graduate student working on BHB/M graduated and moved to a Ph.D. program at the University of California Davis. The current graduate student undertaking this project was trained at the University of Minnesota's Department of Surgery Critical Care and Acute Care Surgery unit. A considerable amount of time was spent on standardizing the surgical protocol to a reproducible level. Minor changes have been made to the surgical protocol utilized by Klein *et al* [1]. One of the modifications included a change in anesthetic and a reduction in the amount (IU) of heparin administered to the rats. The use of ketamine/xylazine as anesthetic agents was changed to isoflurane for two reasons: 1) operated animals recover faster from inhaled versus fixed anesthetics [2]; 2) in the hemorrhagic scenario calculating doses accurately is a challenge and xylazine can be potentially toxic [3]. The dose for whole-animal heparinization was reduced because it was found that 10 IU are sufficient to achieve proper anticoagulatory properties.

After the surgical protocol was standardized, we were not observing deaths following 60% blood loss in the rat model of hemorrhagic shock; animals hemorrhaged to 60% of their calculated blood volume that were not resuscitated with either BHB/M or a blood transfusion still showed prolonged survival (<24 hrs). This is probably attributed to the change from a fixed anesthetic (ketamine/xylazine) to an inhaled one (Isoflurane). For that reason, the surgical protocol had to be further modified. The major changes include the use of a new formula for calculating blood volume, a different endpoint for the first hemorrhagic period, and a reduction in the volume infused.

Calculated Blood Volume

The first modification was the formula used for calculating blood volume. In the past, total blood volume was calculated as 6% of the total body mass. In the literature,

total blood volume in the rat can range from 4.3 to 8% of the total body mass [4]. No blood return (acute) and no therapy (control) groups were performed first. These animals were hemorrhaged to 60% of the calculated blood volume (Figure 1) but were not resuscitated with either a therapeutic infusion or a blood transfusion. Based on the results of Klein et al. [1], we were expecting an average survival of less than one hour. However, we observed an average survival of 1028 minutes (~17 hours) (Figure 2). Based on this observation, it was deemed necessary to re-evaluate the blood volume calculation. We then explored the most common formula used, where total blood volume is calculated as 7% of the total body mass [4, 5]. With this formula, the average survival was 0.5 minutes. Neither of these survival time extremes were suitable for our experiments. For that reason, a blood volume calculation of 6.5% of the total body mass was also explored. Results (mean survival of 6.7 minutes) were not that different from those obtained with 7% blood loss. Finally, we decided to use a formula that accounted for allometric differences because smaller animals have a greater blood volume relative to their body mass than larger animals [4, 6]. The formula was the following:

$$\text{Blood Volume (mL)} = [\text{Total Body Mass (g)} \times 0.06] + .77 \text{ [4]}$$

Using the Lee and Blaufox [4] formula, we obtained a mean survival of 74.17 minutes for acutely-operated control animals. This result is closer to that of Klein *et al* [1]. This formula was used to calculate blood volume in all further experiments.

First Hemorrhagic Endpoint

Klein *et al* [1] used 40% of the calculated blood volume as the endpoint for the first of two hemorrhagic phases. We have switched from using a calculation to using mean arterial blood pressure (MAP) of 25 mmHg as the determinant of the end of the first hemorrhagic phase. A MAP of 25 mmHg is a close calculation for 40% blood loss (Figure 3). However, it allows individual variation to be accounted for. Using MAP, we are also standardizing blood pressure, a physiological measure of recovery from hemorrhage. The second hemorrhagic endpoint is still the withdrawal of a total 60% of the calculated blood volume.

Volume Infused

Since commercially available small-volume resuscitation fluids are administered as a fast infusion [7, 8], acute surgeries were performed to determine whether there was a difference in survival when administering 4 M D-BHB with 43 mM melatonin as a ten minute bolus only (1 ml/kg) versus the same bolus volume plus a one hour slow infusion (100 µl/hr). Survival was monitored for 24 hours. Survival curves (Figure 4) were compared using a Wilcoxon Test. The test showed no statistical difference between treatments at 24 hours ($p>0.05$). In view of the fact that the objective of this study is the optimization of the formulation and because survival is not enhanced by a sustained infusion prior to blood return, all further experiments will be performed using a bolus only infusion.

Specific Aim 1: Dose Ranging Study of BHB/M Components D-Beta-Hydroxybutyrate, Melatonin, and DMSO in Hemorrhagically Shocked Rats

Our Statement of Work, in the description of Specific Aim 1, shows a table depicting a full-factorial design with 27 treatments that would be utilized to perform our dose ranging study (Table 1). The doses are as follows: high D-BHB = 4 M, medium D-BHB = 2 M, low D-BHB = 0.4 M; high melatonin = 43 mM, medium melatonin = 21.5 mM, low melatonin = 4.3 mM; high DMSO = 30%, medium DMSO = 20%, low DMSO 10%. We decided to begin our study by exploring the low concentration ends of the full factorial table (Treatments 1, 2, and 3) and comparing them with the current formulation of BHB/M (Treatment 18).

Survival

Survival curves were compared after 24 hours (Figure 5A) using a Wilcoxon test. There was a significant difference ($p<0.05$) in survival. Treatment no. 1 (0.4 M D-BHB, 4.3 mM melatonin, 10% DMSO) showed 33% survival; treatment no. 2 (2 M D-BHB, 4.3 mM melatonin, 10% DMSO), 71% survival; treatment no. 3 (4 M D-BHB, 4.3 mM melatonin, 10% DMSO), 78% survival; and treatment no. 18 (4 M D-BHB, 43 mM melatonin, 20% DMSO), 80% survival.

Survival curves were also compared from post-operative days 2 to 9 (Table 2). Statistical differences were observed at days 2 and 3 ($p<0.05$), but there were no differences between treatments no. 3 and no. 18. Both of these treatments contain 4 M D-BHB and show the best survival. Ten days after surgery, treatment no. 1 showed 33% survival; treatment no. 2, 29%; treatment no. 3, 44%; and treatment no. 18, 50% (Figure 5B). The test showed no statistical difference between treatments at day 10 ($p>0.05$). It is possible that differences between treatments in later days could not achieve statistical significance because power decreased with time due to a decrease in sample size as a result of death.

These results show that the therapeutic effects of melatonin and DMSO can still be observed at low concentrations. This is a reasonable assumption since serum melatonin peaks in rats are ~8.61×10^{-7} mM [9] and DMSO alone does not provide a therapeutic benefit [1]. Despite not observing a statistical difference at day 10, we do perceive a D-BHB dose-dependent trend (Figure 6). It is possible that this observation is due to the differences in osmolarity between treatments since hyperosmolar solutions increase plasma volume and organ perfusion [7, 8, 10-12] and/or to the glucose sparing effect of D-BHB [13-16]. For this reason, we suggest fixing the concentration of D-BHB at 4 M for all further experiments.

Blood Gas Data

Blood samples were collected throughout the surgical protocol (Figure 1) and analyzed in a blood gas analyzer (BGA) ABL815 Flex (Radiometer America). One-way ANOVAs with Tukey's *post hoc* test were performed to find treatment differences within different time points for total hemoglobin (tHB), hematocrit (Htc), pH, pressure of carbon dioxide (pCO_2), pressure of oxygen (pO_2), saturation of oxygen (sO_2), potassium ion (K^+), sodium ion (Na^+), calcium ion (Ca^{++}), chloride ion (Cl^-), glucose (Glu), and lactate (Lac). No differences where found at any time point for pH, pCO_2, pO_2, sO_2, Cl^-, Glu and Lac.

There were differences in tHb after bolus infusion, after 60% blood loss, and one hour after 60% blood loss between treatment no. 1 (0.4 M D-BHB, 4.3 mM melatonin, 10% DMSO) and treatment no. 18 (4 M D-BHB, 43 mM melatonin, 20% DMSO) (p<0.05). These differences can be attributed to the differences in D-BHB concentration. Hyperosmolar solutions rapidly mobilize fluids from the intracellular space into the intravascular space, hence causing a hemodilution effect [7, 8, 10-12]. Differences, also between treatment no. 1 and treatment no. 18, were observed for Htc after bolus infusion (p<0.05) and one hour after 60% blood loss (p<0.05). However, our BGA calculates Htc as a function of tHb, so differences observed in tHb are likely to be reflected in Htc as well.

Classic changes in plasma electrolyte concentration during hemorrhagic shock include hyperkalemia [17, 18] and hyponatremia [17]. These correlate with increased intracellular Na^+ [17, 18]. A significant decrease in K^+ concentration at 60% blood loss was present in treatments no. 1 and 18 when compared to treatment no. 2 (p<0.05). Also at 60% blood loss, Na^+ levels were significantly higher in treatment 18 in comparison with treatment no. 1 (p<0.05), treatment no. 2 (p<0.05), and treatment no. 3 (p<0.05). Because the hyperkalemia and hyponatremia in hemorrhagic shock seem to be due to the cells' inability to maintain intracellular/extracellular differences [17], perhaps by infusing the sodium salt form of D-BHB we are able to compensate for such imbalances. Furthermore, through the mechanisms underlying alterations in ion transport during shock have not yet been elucidated, it has been suggested that they occur due to a loss in cellular metabolic energy [19]. D-BHB is catabolized via the Krebs cycle, providing an alternative fuel source [20] and maintaining cellular energy at a steady state and possibly preventing ionic variations from taking place.

Correlation to Survival. Pearson's correlation analyses were performed to elucidate whether survival could be predicted by any of the parameters measured by the BGA. We found no strong correlation between survival and tHb, Htc, pCO_2, pO_2, sO_2, K^+, Na^+, Ca^{++}, Cl^-, or Glu. Lac showed a slight negative correlation (R=-0.6138, p<0.01) to survival. This is consistent with previous reports suggesting that improved acid-base balance prolongs short-term survival [21, 22].

PowerLab Data

Physiological parameters such as mean arterial blood pressure (MAP; Figure 7), heart rate (Figure 8), and temperature (Figure 9) were monitored during the whole procedure using a PowerLab 30/4 (ADInstruments). One-way ANOVAs with Tukey's *post hoc* test were performed to find treatment differences within the time points outlined in Figure 1. The only statistical difference ($p<0.05$) observed was in body temperature between treatments 2 and 18 after 60% blood loss. We do not have an explanation for this difference, but it does not seem to be of importance as it does not hold in later time points.

Correlation to Survival. As with BGA data, correlations between PowerLab data and survival were performed with the objective of clarifying whether MAP, heart rate, or temperature could forecast survival. No strong correlation was observed between survival and MAP, heart rate, or temperature during the entire surgical procedure (Figure 1).

Plasma D-BHB

Blood samples were collected during the surgical protocol (Figure 1). Shed blood was also sampled. Samples were spun down to obtain plasma. Plasma was analyzed for D-BHB levels using a colorimetric kit (Stanbio Laboratories). Data obtained was analyzed using a one-way ANOVA with Tukey's *post hoc* test to discriminate treatment variation. Statistical differences were observed after bolus infusion between treatments 1 and 18 ($p<0.05$); between treatments 1 and 18 ($p<0.05$) and treatments 1 and 3 ($p<0.05$) after 60% blood loss; and between treatments 1 and 18 ($p<0.05$) in shed blood (Figure 10). Even though treatment 1 managed to achieve a 3-fold increase from baseline in D-BHB plasma levels after bolus infusion, it was not successful at maintaining such levels for an extended period of time (Figure 11). These observations are concordant with the dose-ranging study performed.

Correlation to Survival. Plasma D-BHB data was analyzed using Pearson's correlations to identify a relationship between plasma D-BHB and survival. No strong correlation was observed based on the measurements made throughout the surgical procedure.

Key Research Accomplishments

- Purchased equipment for the funded project W8IXWH-11-1-0409
- Standardization and reproducibility of hemorrhagic protocol in a rat model
- Initiation of full-factorial design
- Exploration of the low concentration end of the full-factorial design (Treatments 1, 2, and 3)
- Determination that 4 M D-BHB is the ideal therapeutic concentration

Reportable Outcomes

The ultimate goal of this research project is to deliver a portable therapy for hemorrhagic shock to soldiers in harms way. We took a major step toward making this blood loss therapy available to military personnel by signing a license agreement with Ariel Pharmaceuticals, a private, specialty pharmaceutical company based in Broomfield, CO. Ariel intends to file an IND application with the FDA and commence clinical trials in the second half of 2012 (see press releases from Ariel and the University of Minnesota in Appendices).

Conclusion

The advances in the proposal submitted in the Statement of Work are confined to Specific Aim 1. This is coherent with the time table provided in that document. The low concentration end of the full-factorial design was explored. Results obtained suggest that treatments #3 and #18 prolong short-term (3 day) survival compared to treatment #1. No statistical differences were observed in long-term (10 day) survival. However, this is possibly due to insufficient power. From our data and that of Klein *et al* [1], it seems evident that the organism requires a high concentration of a fuel source in order to overcome the negative repercussions of hemorrhagic shock.

Regarding melatonin, there being no statistical difference between treatments 3 and 18, it is possible that its therapeutic effect can be observed at concentrations lower than 4.3 mM.

BGA data suggests that the infusion of D-BHB may contribute to maintain intracellular/extracellular fluid differences as observed in the plasma levels of K^+ and Na^+. Furthermore, correlation analyzes demonstrate that acid-base imbalances, in terms of Lac concentration, negatively affect survival. D-BHB may maintain Lac levels at a minimum by sparing glucose utilization [13-16], hence attenuating Lac production.

Physiological data such as MAP, heart rate, and temperature did not provide information that helped elucidate treatment differences or predict survival. However, their monitoring is of great importance throughout the surgical protocol (e.g. MAP needs to me observed for the endpoint of the first hemorrhagic period) and will continue to be monitored.

Measured plasma D-BHB levels were inconsequent in predicting survival. Differences between treatments were observed, however, these differences were expected because they were consistent with the different concentrations infused.

So What?

Evaluating the low concentration end of the full factorial design allowed for the identification of 1) whether D-BHB concentration had an effect on survival, and 2) whether the therapeutic effects of melatonin could be observed at low concentrations. We know from previous studies that DMSO has no beneficial value in cases of hemorrhagic shock [1]. We do observe a D-BHB dose-dependent trend in 10 day survival (Figure 6), for that reason we suggest fixing D-BHB concentration at 4 M and not exploring any treatments in the full factorial table (Table 1) that have a lower molarity. Also, melatonin favorably effects survival even at low concentrations as observed by the absence of statistical difference in survival between treatments 3 and 18. Because 4.3 mM was the lowest melatonin concentration included in our Statement of Work, we see the need to modify our proposed factorial design. We suggest the fixation of not only D-BHB but also DMSO concentrations. DMSO is used as a solvent of melatonin, therefore the lower the melatonin concentration, the lower the need for DMSO. For experimental reproducibility, we suggest fixing DMSO concentration at 2%. Regarding melatonin, we see the need for a dose-ranging study that explores even lower concentrations than 4.3 mM. Consequently, we move for the absolute modification of the treatments for Specific Aim 1. The five proposed treatments are shown in Table 3 and include: 1) 4 M D-BHB, 4.3 mM melatonin, 2% DMSO, 2) 4 M D-BHB, 2.15 mM melatonin, 2% DMSO, 3) 4 M D-BHB, 0.43 mM melatonin, 2% DMSO, 4) 4 M D-BHB, 0.043 mM melatonin, 2% DMSO, and 5) 4 M D-BHB, no melatonin, 2% DMSO.

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Figures

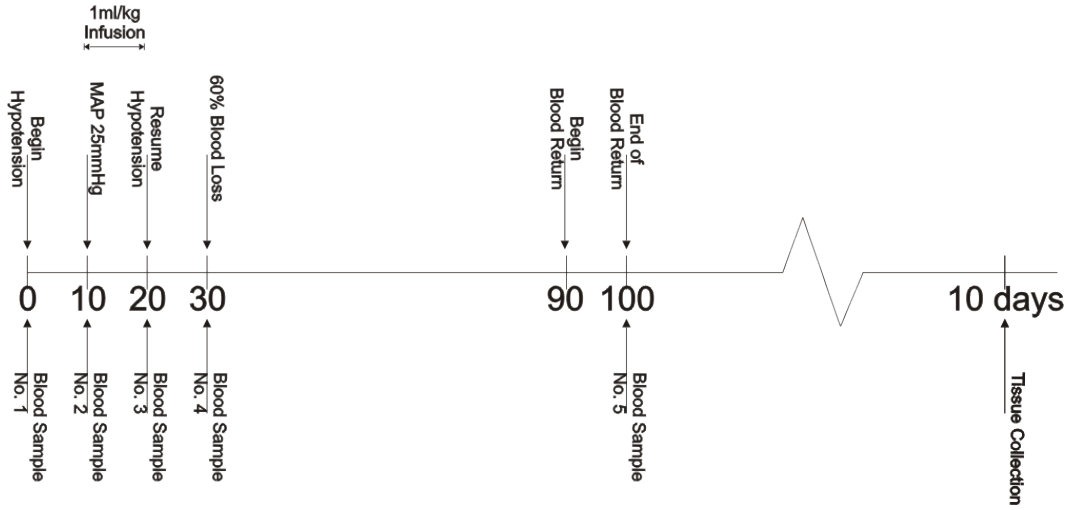


Figure 1. Timeline depicting current hemorrhagic protocol. Time is listed in minutes unless otherwise stated. Above the line are surgical endpoints. Below the line are sampling times.

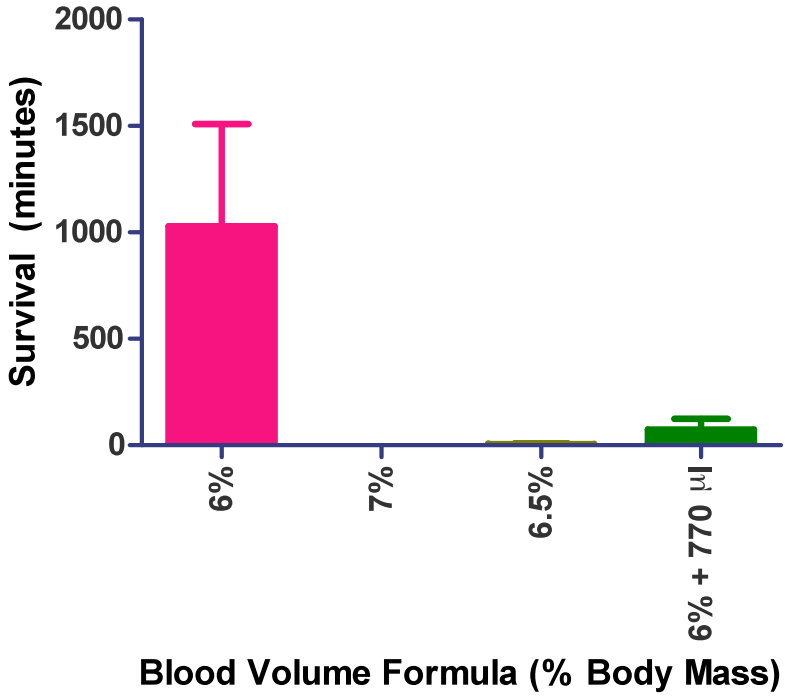


Figure 2. Mean survival using different formulas to calculate total blood volume. Error bars are Standard Errors. Sample sizes are as follows: 6%, n=5; 7%, n=3; 6.5%, n=3; and 6% + 770 µl, n= 6.

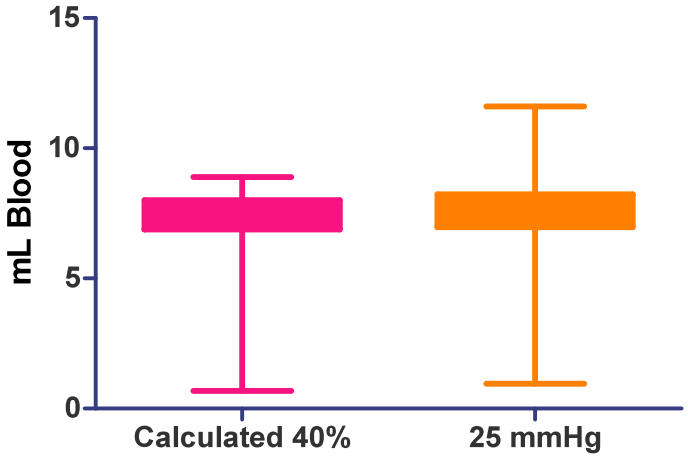
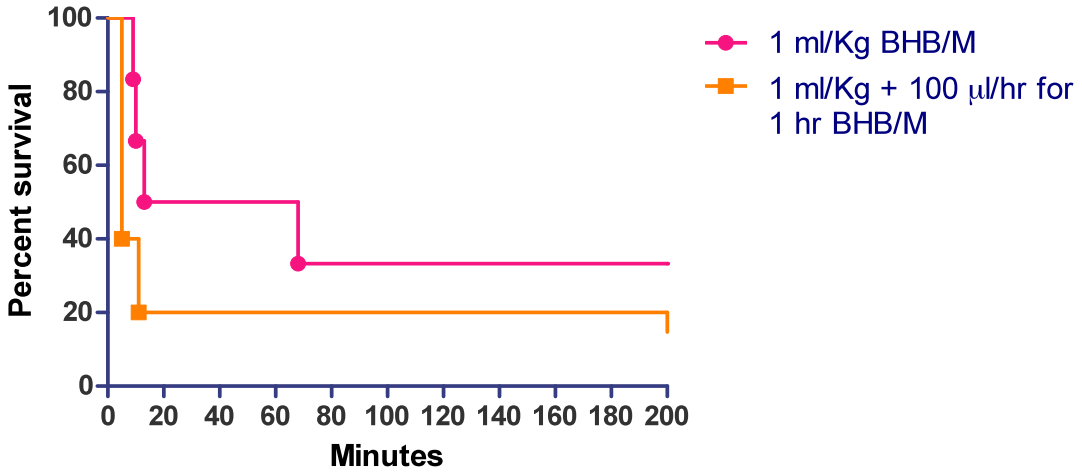


Figure 3. Mean volume (mL) of blood calculated as 40% of the total blood volume versus blood withdrawn to achieve 25 mmHg for the same animals (n=72). Error bars are ranges. Ranges for "Calculated 40%" blood loss are due to differences in body weight which range from 254.6 g to 370.8 g. Ranges for "25 mmHg" are due to the differences in volume required to achieve MAP of 25 mmHg.

A



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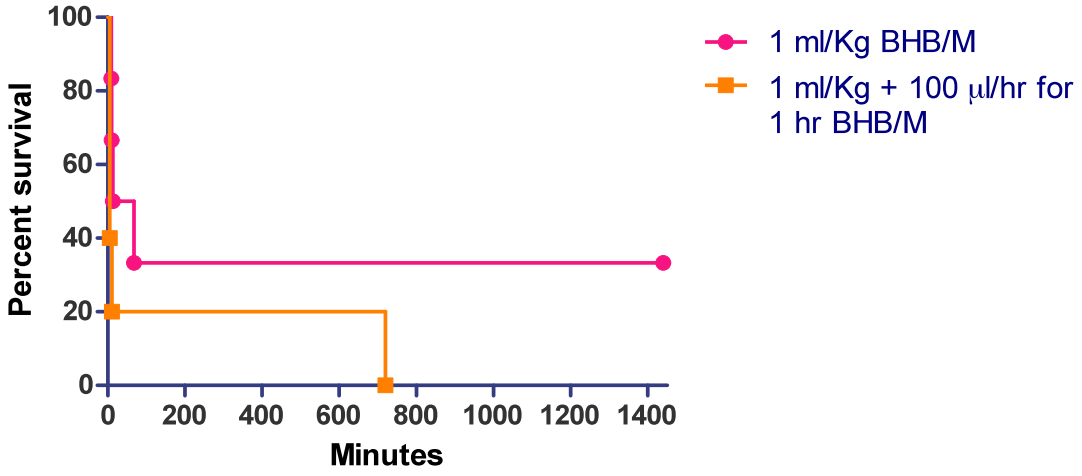
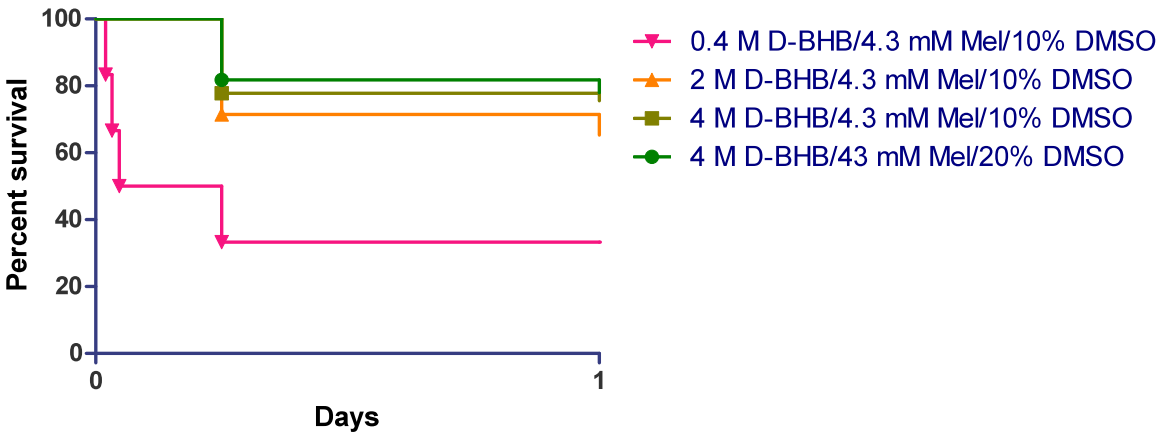


Figure 4. *A.* Kaplan-Meier survival plot showing the first 200 minutes of the 24-hour period. *B.* Kaplan-Meier survival plot comparing the effects of BHB/M as a bolus only versus a bolus plus slow infusion over a 24-hour period. Sample sizes are as follows: BHB/M bolus, n = 6; BHB/M bolus plus slow infusion, n = 5.

A



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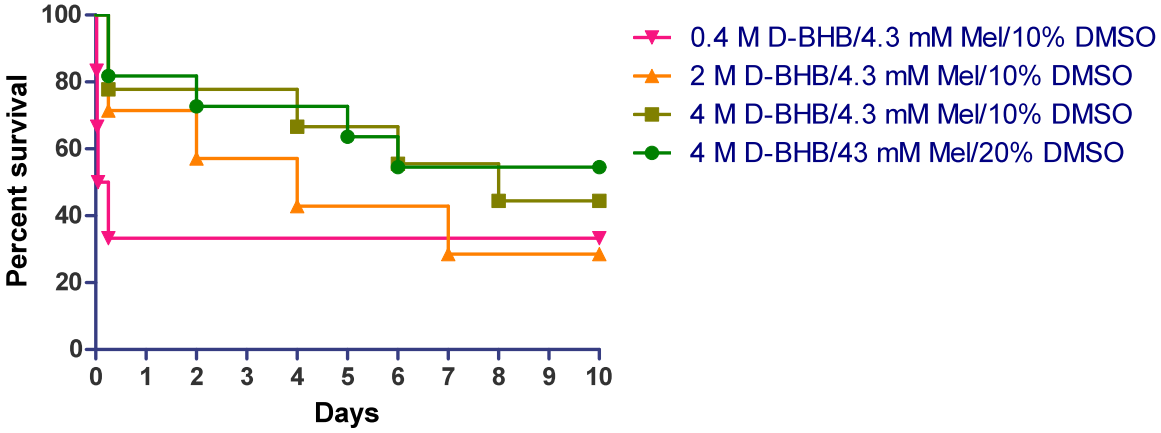

Figure 5. Kaplan-Meier survival plots comparing the effects of Treatments 1, 2, 3 and 18 from the full factorial table. *A.* First day of the observation period. *B.* All ten days of the observation period. Treatment 3 = 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (n = 9); Treatment 2 = 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (n = 9); Treatment 1 = 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (n = 7); Treatment 18 = 4 M D-BHB / 43 mM Melatonin / 20% DMSO (n = 10).

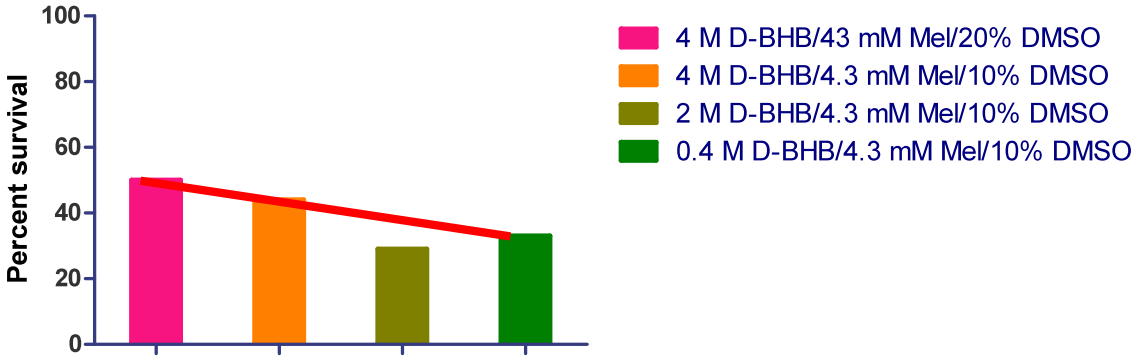

Figure 6. Percent survival at 10 Days. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10. Red line indicates trend.

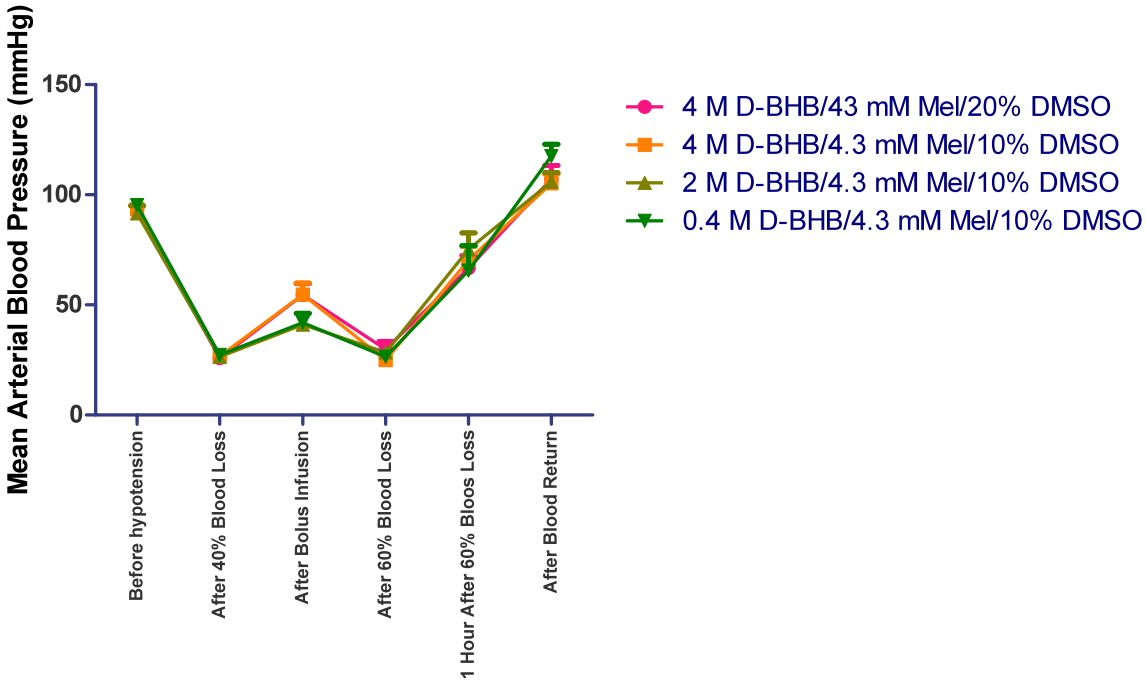

Figure 7. Mean Arterial Blood Pressure throughout the surgical protocol. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10.

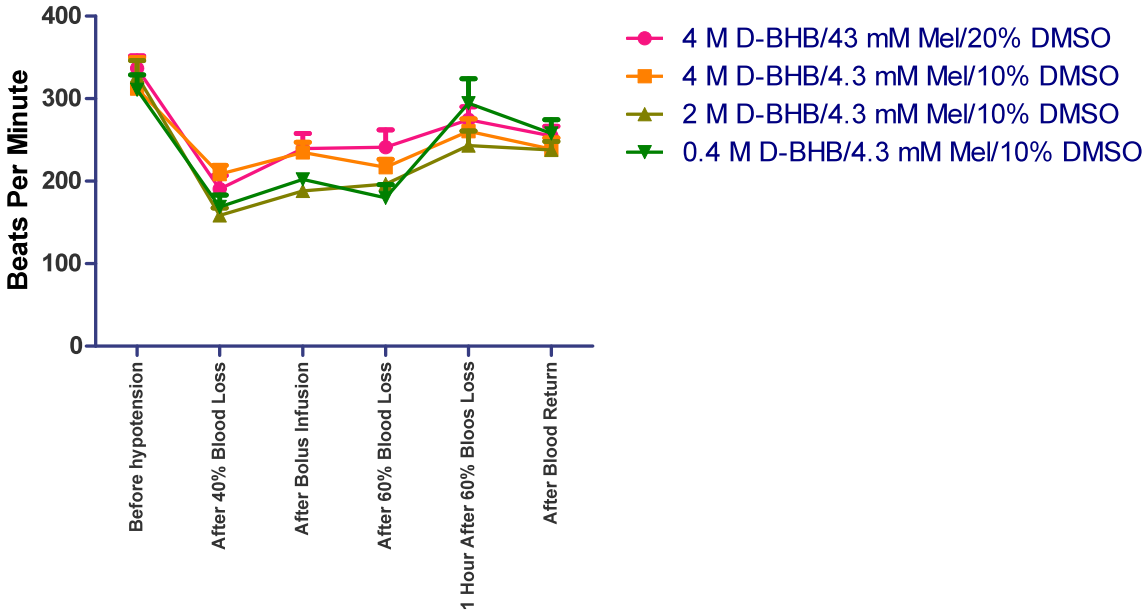

Figure 8. Heart rate (Beats Per Minute) throughout the surgical protocol. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10.

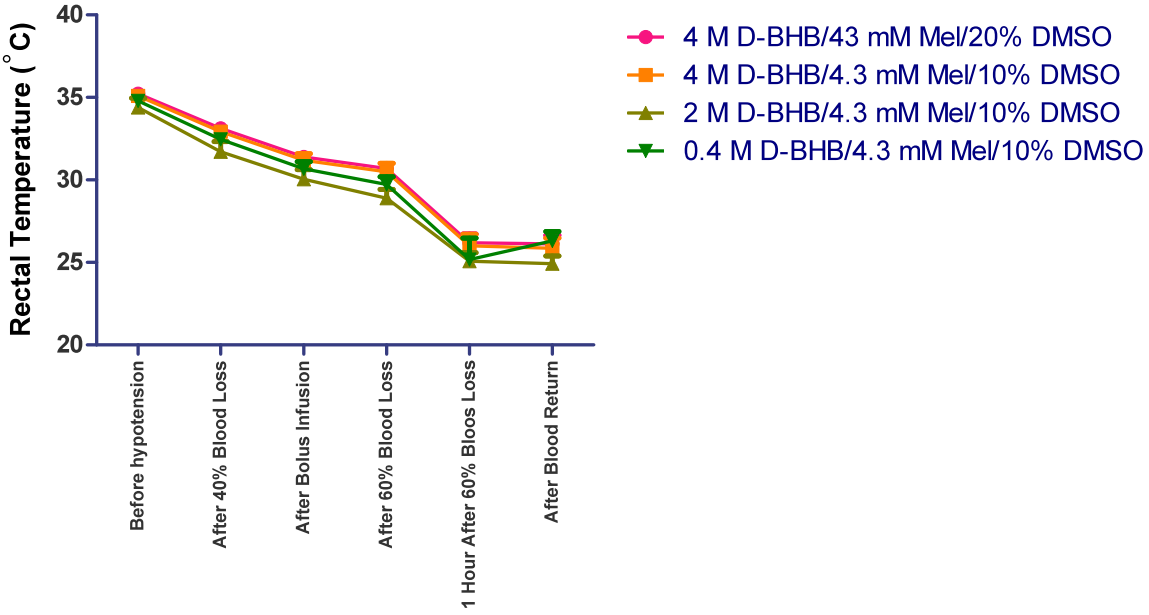

Figure 9. Temperature (°C) throughout the surgical protocol. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10.

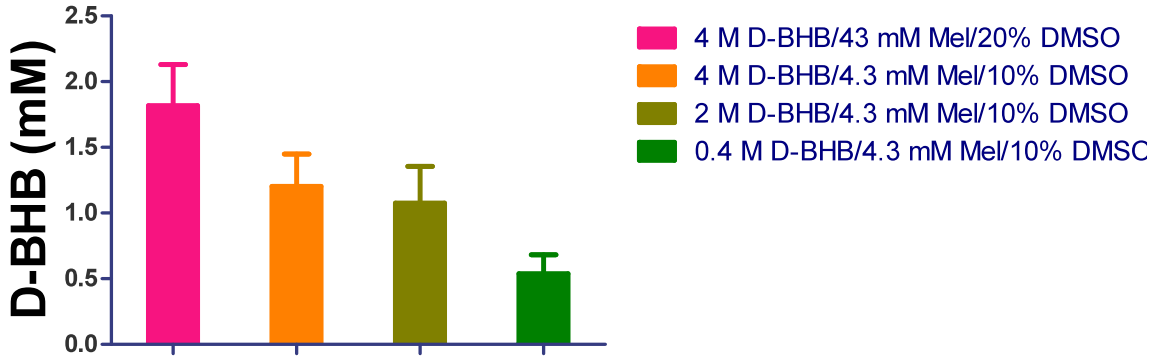

Figure 10. D-BHB plasma levels in shed blood. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10.

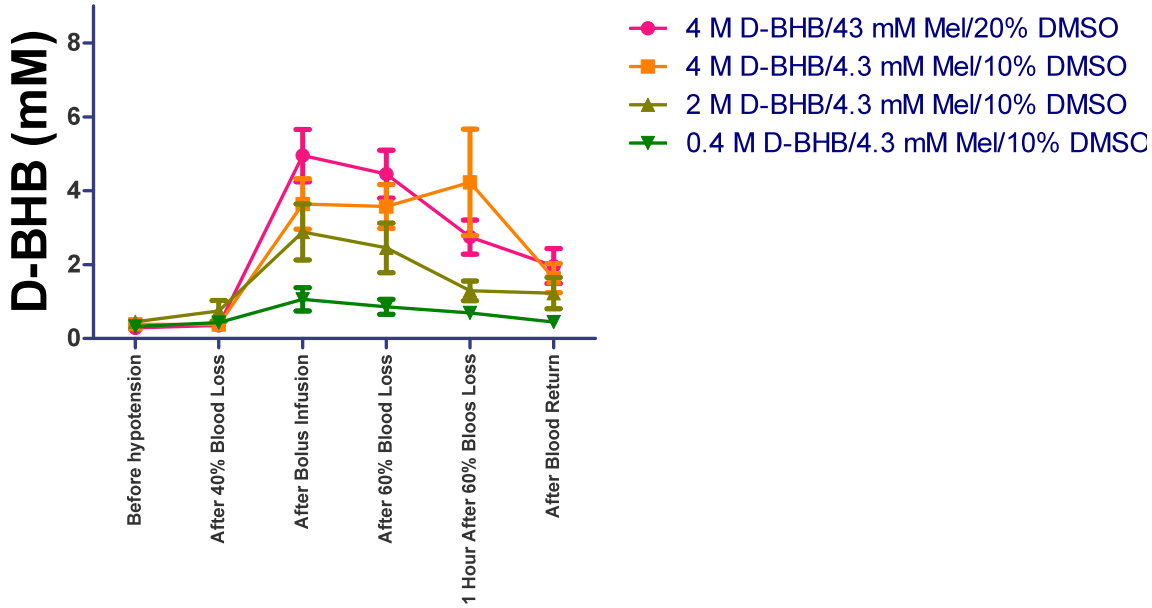

Figure 11. D-BHB plasma levels throughout surgical protocol. Error bars are Standard Errors. 4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 3), n = 9; 2 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 2), n = 6; 0.4 M D-BHB / 4.3 mM Melatonin / 10% DMSO (Treatment 1), n = 7; 4 M D-BHB / 43 mM Melatonin / 20% DMSO (Treatment 18), n = 10.

Tables

Table 1. Full factorial table take from previously submitted Statement of Work. Concentrations are as follows: High BHB = 4 M, Med BHB = 2 M, Low BHB = 0.4 M; High Melatonin = 43 mM, Med Melatonin = 21.5 mM, Low Melatonin = 4.3 mM; High DMSO = 30%, Med DMSO = 20%, Low DMSO = 10%.

Ex. Gp.	D-BHB	Melatonin	DMSO	Ex. Gp.	D-BHB	Melatonin	DMSO	Ex. Gp.	D-BHB	Melatonin	DMSO
1	Low	Low	Low	10	Low	Low	Med	19	Low	Low	High
2	Med	Low	Low	11	Med	Low	Med	20	Med	Low	High
3	High	Low	Low	12	High	Low	Med	21	High	Low	High
4	Low	Med	Low	13	Low	Med	Med	22	Low	Med	High
5	Med	Med	Low	14	Med	Med	Med	23	Med	Med	High
6	High	Med	Low	15	High	Med	Med	24	High	Med	High
7	Low	High	Low	16	Low	High	Med	25	Low	High	High
8	Med	High	Low	17	Med	High	Med	26	Med	High	High
9	High	High	Low	18	High	High	Med	27	High	High	High

Table 2. Percent survival, p-values, and statistical power per day.

Post-surgical Day	Treatment	Survival	p-value	Power
10	4 M BHB / 43 mM Mel / 20% DMSO	50%	0.1928	0.2012
	4 M BHB / 4.3 mM Mel / 10% DMSO	44%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	29%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
9	4 M BHB / 43 mM Mel / 20% DMSO	50%	0.1928	0.2192
	4 M BHB / 4.3 mM Mel / 10% DMSO	44%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	29%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
8	4 M BHB / 43 mM Mel / 20% DMSO	50%	0.1928	0.2420
	4 M BHB / 4.3 mM Mel / 10% DMSO	44%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	29%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
7	4 M BHB / 43 mM Mel / 20% DMSO	50%	0.1578	0.2619
	4 M BHB / 4.3 mM Mel / 10% DMSO	56%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	29%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
6	4 M BHB / 43 mM Mel / 20% DMSO	50%	0.1561	0.2990
	4 M BHB / 4.3 mM Mel / 10% DMSO	56%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	43%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
5	4 M BHB / 43 mM Mel / 20% DMSO	60%	0.0961	0.3254
	4 M BHB / 4.3 mM Mel / 10% DMSO	67%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	43%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
4	4 M BHB / 43 mM Mel / 20% DMSO	70%	0.0719	0.3488
	4 M BHB / 4.3 mM Mel / 10% DMSO	67%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	43%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
3	4 M BHB / 43 mM Mel / 20% DMSO	70%	0.0485	0.3785
	4 M BHB / 4.3 mM Mel / 10% DMSO	78%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	57%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
2	4 M BHB / 43 mM Mel / 20% DMSO	70%	0.0485	0.4313
	4 M BHB / 4.3 mM Mel / 10% DMSO	78%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	57%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		
1	4 M BHB / 43 mM Mel / 20% DMSO	80%	0.0276	0.5301
	4 M BHB / 4.3 mM Mel / 10% DMSO	78%		
	2 M BHB / 4.3 mM Mel / 10% DMSO	71%		
	0.4 M BHB / 4.3 mM Mel / 10% DMSO	33%		

Table 3. Proposed new treatments for Specific Aim 1.

BHB (M)	Mel (mM)	DMSO
4	4.300	2%
4	2.150	2%
4	0.430	2%
4	0.043	2%
4	0.000	2%

New U of M startup may save lives of victims of massive blood loss and trauma

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MINNEAPOLIS / ST. PAUL (10/18/2011) — A new technology from the University of Minnesota has resulted in a startup that may help prolong the lives of victims suffering from massive blood loss or trauma. The Office for Technology Commercialization has signed a license agreement with Denver-based Ariel Pharmaceuticals authorizing the private company to develop and commercialize the therapy.

Researchers at the Twin Cities and Duluth campuses — including surgeon Gregory Beilman, biologist Matthew Andrews and biomedical scientist Lester Drewes — designed a low-volume resuscitation fluid that may increase the survival rates of people who die from hemorrhagic shock. They developed the therapy, called Tamiasyn, based on their studies of the biological process of hibernation in ground squirrels (gophers).

"We're excited to have the opportunity to further develop the technology and we're confident this unique therapeutic approach will provide patients with a better chance of survival," said Steve Orndorff, president and CEO of Ariel Pharmaceuticals.

The technology could offer first responders, emergency department staff and military medics a simple, safe and reliable product that prevents life-threatening complications due to severe blood loss. At the same time, it could help prevent organ damage during resuscitation.

"Licensing with a private pharmaceutical company is the next step in bringing this drug to the marketplace and, more importantly, in bringing this drug first into the hands of the military personnel in harm's way," said Andrews. "Ariel Pharmaceuticals' expertise will shepherd our proposed therapy through the clinical approval to the point that our research begins to save lives."

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The mission of the University of Minnesota's Office for Technology Commercialization is to translate university research into new products and services that provide growth opportunities for its licensees,

benefit the public good, improve the quality of life, and generate revenue to support research and education goals.

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Ariel Pharmaceuticals, Inc. Acquires Novel Compound for Hemorrhagic Shock from the University of Minnesota

Ariel to File Investigational New Drug (IND) Application with FDA, Commence Clinical Trials in 2012

Broomfield, CO and Minneapolis, MN October 18, 2011 – Ariel Pharmaceuticals, Inc., a private, specialty pharmaceutical company focused on the development and commercialization of products for acute central nervous system (CNS) diseases and trauma, announced today that it has in-licensed Tamiasyn™ (AP-1100) for the treatment of hemorrhagic shock from the University of Minnesota.

"We are excited to have the opportunity to further develop Tamiasyn and are confident the unique neuroprotective, cytoprotective and cardioprotective attributes of this product will be more effective than the resuscitation fluids currently available," said Steve Orndorff, President and CEO of Ariel Pharmaceuticals. "Given the dire patient need and the lack of any improvements in this field in more than 20 years, we're dedicated to advancing Tamiasyn through commercialization as a treatment for hemorrhagic shock to provide patients with a better chance of survival."

According to the National Center for Health Statistics, hemorrhagic shock is the leading cause of death in trauma patients worldwide, with primary causes including motor vehicle accidents (MVA) and gunshot wounds (GSW). Approximately 80,000 people die from MVA and GSW each year in the U.S. Estimates establish a global market in excess of $1 billion.

Tamiasyn is a new drug therapy in a proprietary, hypertonic formulation comprised of various therapeutic agents that readily cross the blood-brain barrier. Administered intravenously to hemorrhaging patients and having demonstrated neuroprotective and cytoprotective efficacy, Tamiasyn is a significant improvement over current therapies that only improve oxygen availability or restore blood volume.

Studies at the University of Minnesota in trauma animal models have shown that Tamiasyn stabilizes organs, including the brain; reduces oxidative stress or

damage to tissues; reduces reperfusion injury and reduces the buildup of toxic levels of lactic acid while sparing energy use such as ATP.

Lester R. Drewes, Ph.D., a professor at the University of Minnesota and a biomedical scientist, explained that, "Our preclinical research has shown that Tamiasyn (AP-1100) is effective when administered after hemorrhagic shock in both small and large animal models. Therefore, Tamiasyn (AP-1100) could provide value in minimizing tissue and brain damage due to hemorrhagic shock in military combat, auto accidents or gunshot wounds where there is evidence of trauma."

Tamiasyn has been tested for efficacy in treating small and large animals subjected to acute blood loss. The results of a small animal model of hemorrhagic shock showed that administration of Tamiasyn significantly increased survival in rats subjected to 60% blood loss as compared to controls. No significant difference in survival was found between Tamiasyn treated animals and sham operated animals that were not subjected to blood loss.

Ariel intends to file an IND application with the FDA and commence clinical trials in the second half of 2012.

About Hemorrhagic Shock

Hemorrhagic shock occurs when blood loss exceeds the body's ability to compensate, leading to vascular collapse, inadequate vital organ perfusion, insufficient oxygenation and lack of nutrients necessary for normal cellular function. According to the National Center for Health Statistics, hemorrhagic shock is the leading cause of death in trauma patients worldwide, with primary causes including motor vehicle accidents (MVA) and gunshot wounds (GSW). Approximately 80,000 people die from MVA and GSW each year in the U.S.

About Ariel Pharmaceuticals, Inc.

Ariel Pharmaceuticals, Inc. is a private, specialty pharmaceutical company focused on the development and commercialization of drugs that improve quality of life for patients with acute neurological disorders and trauma. Ariel is developing three drugs intended to meet unmet medical needs in migraine, hemorrhagic shock and post-operative cognitive decline. The company's business strategy is based on in-licensing clinic-ready drugs in acute care clinical indications that have well-defined efficacy endpoints and where there is little to no competition in order to enable Ariel to rapidly build shareholder value. For more information, please visit: www.arielpharma.com

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