Prophylactic and therapeutic beta-hydroxybutyrate improved motor dysfunction and cortical damage in a mouse model of traumatic brain injury

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Introduction

Over 1.7 million traumatic brain injuries (TBIs) are documented yearly in the United States alone due to their association with hospitalization. This figure, though, likely underestimates the true number of TBIs as many "mild" injuries go unreported. Even lower-grade TBIs can negatively impact brain health, affecting motor coordination, cognition, tissue integrity, and potentially leading to post-concussive syndrome, with few therapies available to mitigate these effects. Beta-hydroxybutyrate (BHB) is a potential therapeutic option, as it may serve as an alternative energy source when glycolysis is impaired during the acute phase after TBI. Despite promising insights into BHB, questions remain about its safety, efficacy, optimal dosage, timing, and long-term effects, particularly before integrating it into standard TBI care. This study aimed to determine the ideal timing and treatment regimen for BHB in a closed-head injury mouse model of TBI. We hypothesized that intranasal administration of BHB would improve TBI-induced motor dysfunction and reduce TBI-induced expression of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), a measure of brain damage.

Methods

Animals. Adult, female C57BL/6J mice (Jackson Labs, Inc.; Bar Harbor, ME) were housed in groups of 4-5 on a 12h:12h light schedule, and standard water and chow were provided ad libitum for the entire study. The ordering and receipt of the animals were coordinated with the study design that called for 3 days of beam walk training before injury and injury induction at the age of 12 weeks. The average weight of the animals at induction was 21.9 ± 1.8 grams. There was a total of 120 mice randomized by weight into an N=20 per group. All animal procedures were approved by the Institutional Animal Care and Use Committee at Inotiv-Boulder, Inc. At the time these experiments were conducted, only female mice had been validated.

Study Design. A pilot study using liquid chromatography/mass spectroscopy (N=3) determined levels of BHB in the plasma and brain at baseline and at 10, 30, 60, 120 and 180 minutes following a single intranasal dose of BHB [R-(-)-3-hydroxybutryic acid sodium salt; Millipore-Sigma, Cat #298360; 30 mg/kg in sterile, 0.9% saline; 25 µl dose volume].

During the experimental phase, the treatments were provided as two, 25-µl intranasal doses (one per nostril). In accordance with the brain absorption profile of BHB, all animals received pretreatments of either saline or BHB (50 or 100 mg/kg) 30 minutes directly before sham or TBI induction. Directly after sham or TBI induction, the animals were either maintained on daily doses of either saline only (sham and TBI groups), switched to saline alone (Preinjury; Pre), or switched to, or maintained on, BHB treatments (Postinjury; Post, Pre + Post, and 2x Pre + Post). This dosing regimen resulted in daily BHB doses of either 0 (Sham and TBI), 50 (Post, Pre + Post), or 100 (2x Pre + Post) mg/kg/d.

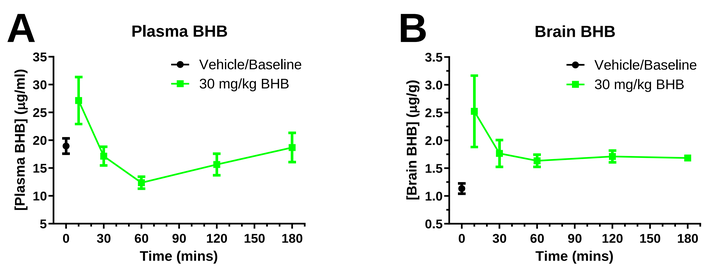
Weight-Drop Injury. All mice were anesthetized with 2.5% isoflurane delivered for 2 min. They were then placed individually in the prone position on a foam bed with their heads directly under a Plexiglas tube. A brass weight (80 g) was dropped once through the tube from a height of 0.8 m, striking the head directly and resulting in a closed-head injury. Animals were moved right after being struck by the weight so that no secondary impacts occurred and were given buprenorphine (0.1 mg/kg SC). Sham-injured mice underwent the same procedure, but the weight was not released. All mice were placed in a heated recovery cage and monitored until ambulatory before being returned to their cage. Sham-injured mice recovered the righting reflex at 51.9 ± 4.5 sec, while injured mice had average righting reflex recovery times of >2 min. Daily monitoring indicated that all animals that survived induction had no unexpected changes in water consumption, body weight or home cage behavior.

Beam Walking. Animals were trained to a narrow plastic beam (5 mm wide x 1m long) that was suspended 50 cm above a table with a goal box at the end. The animal was placed on the beam and the number of foot-faults for the right hindlimb was recorded over 50 steps. Three days of preinjury training (4 trials per day) established baseline performance. The animals were then tested on days 1 and 3 after TBI (1 trial each day) to determine acute motor recovery (N=9-12 per arm).

Post-injury levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1). The cerebral cortex and hippocampus were harvested at 4 hours, 24 hours, and 14 days after TBI (N=5 per group per time point), and the levels of UCH-L1 were determined by ELISA (Abcam PLC; Cat #ab235641). UCHL1 was assayed in cell extraction buffer according to the manufacturer's specifications. Samples, standards, and antibody cocktail were added to separate wells of a 96-well plate and incubated at ambient temperature for 1 hour with agitation followed by a wash cycle. TMB development solution was then added to each well and incubated at room temperature for 10 min with agitation and protected from light. Finally, stop solution was added to each well and absorbance was read at 450 nm.

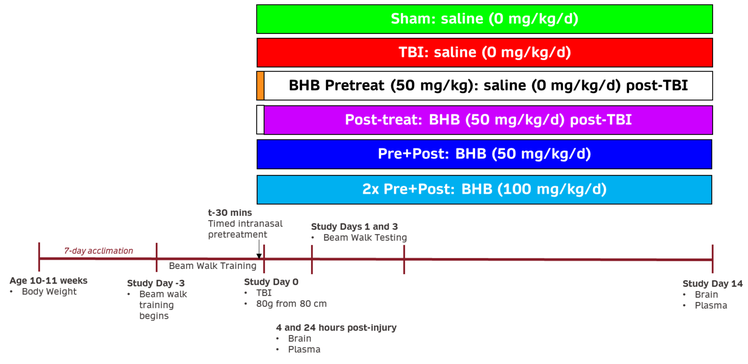
Statistics. Statistical analyses were performed using GraphPad Prism 10.1.1 (GraphPad Software, Inc.). Two-way ANOVA (time x treatment) was used to determine main effects, followed by Tukey's post hoc test to detect significant group differences. Statistical significance was set at $p < 0.05$. If a significant interaction term was detected, each time point was analyzed separately by one-way ANOVA followed by Tukey's post hoc test.

1 BHB levels in plasma and brain after intranasal dosing



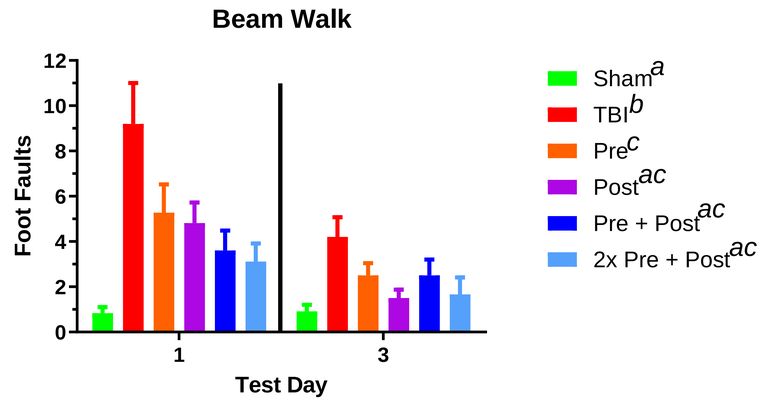
Pilot dosing data for a single dose of BHB (30 mg/kg) and resulting BHB levels in the plasma (A) and brain (B) over a 180-minute time course. BHB levels (*green squares*) peaked over baselines (*black circles*) at 10 minutes after administration in both tissues. Plasma BHB then returned to the equivalent of baseline by 30 minutes, but brain levels of BHB remained elevated for up to 180 minutes. Error bars represent the standard error of the mean (SEM). N= 3 per group at each time point.

2 Experimental study design



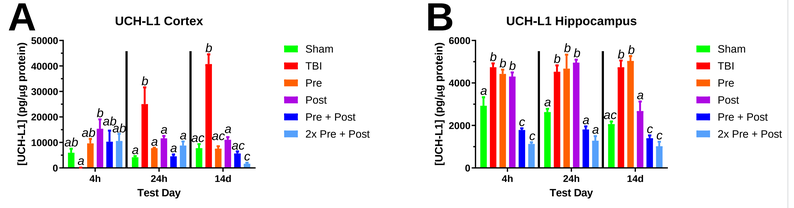
Animals were acclimated for 7 days and then trained on the beam walk for 3 days. After the last beam walk training, animals received saline (0 mg/kg) or BHB (50 or 100 mg/kg) 30 minutes before the induction of TBI. The shams, untreated TBI controls, and pretreatment only groups received saline (0 mg/kg/d) once a day for the remainder of the study, while the post-treatment (50 mg/kg/d), pre + post-treatment (50 mg/kg/d), and 2x pre + post treatment (100 mg/kg/d) groups received BHB for the remainder of the study. Brains and plasma were harvested from 5 animals from each group at 4 and 24 hours after TBI. The remaining animals were then tested on the beam walk at 1 and 3 days post injury. On study 14, brains and plasma were harvested from all remaining animals.

3 BHB reduced foot faults in the beam walk after TBI



All treatments resulted in significantly fewer foot faults during the beam walk compared to untreated TBI animals [$F_{(5,112)}$= 10.0, p<0.001]. Error bars represent the SEM. N= 9-12 per group. Data sets that do not share common letter designations differed significantly in Tukey's *post hoc* test.

4 BHB lowered brain levels of UCH-L1 after TBI



TBI increased UCH-L1 levels in both the cortex (A) and hippocampus (B), but the effects of BHB varied between these brain regions, with significant interaction terms observed. A one-way ANOVA at each time point was used to assess the main effects of treatment and significant group differences. In the cortex, all treatments significantly lowered UCH-L1 levels, while in the hippocampus, the Pre + Post treatments were most effective in reducing UCH-L1. Error bars represent the SEM, with N=5 per group per time point. Data sets not sharing common letter designations differed significantly in Tukey's *post hoc* test.

Conclusions

- All intranasal BHB treatment regimens improved motor dysfunction after TBI as measured on the beam walk
- All BHB treatments mitigated TBI-induced increases of UCH-L1 in the cerebral cortex
- The hippocampal UCH-L1 data suggest that BHB treatment administered both before and after TBI may provide the best protection from brain damage
- Intranasal BHB may reduce the negative sequelae of TBI with a wide therapeutic window