Supplementary Data

The Animal Research: Reporting *In Vivo* Experiments (ARRIVE) Checklist

Title

1. Provide as accurate and concise a description of the content of the article as possible

Glibenclamide produces region-dependent effects on cerebral edema in a combined injury model of traumatic brain injury (TBI) and hemorrhagic shock (HS) in mice

Abstract

2. Provide an accurate summary of the background, research objectives (including details of the species or strain of animal used), key methods, principal findings, and conclusions of the study

Cerebral edema is critical to morbidity/mortality in TBI and is worsened by hypotension. Glibenclamide may reduce cerebral edema by inhibiting sulfonylurea receptor-1 (Sur1). The effect of glibenclamide on diffuse cerebral edema exacerbated by hypotension/resuscitation is unknown.

We aimed to determine if glibenclamide improved pericontusional and/or diffuse edema in controlled cortical impact (CCI) (5m/sec, 1 mm depth)+HS (35 min), and compared its effects in CCI alone. C57/BL6 mice were divided into five groups ($n = 10$/group): naïve, CCI+vehicle, CCI+glibenclamide, CCI+HS+vehicle, and CCI+HS+glibenclamide. Intravenous glibenclamide was given 10 min post-injury, followed by a subcutaneous infusion for the experiment duration. Pericontusional and diffuse brain edema in injured and contralateral hemispheres were quantified at 24 h by wet-dry weight. The same protocol was followed for 72 h in the combined injury model CCI+HS ($n = 9$/group).

Ipsilateral (I) edema was greater in CCI+HS (I% brain water [BW] = 80.4% vehicle vs. 78.3% naïve, $p < 0.01$), but not reduced by glibenclamide (I%BW = 80.4%). Ipsilateral edema also developed in CCI alone (I%BW = 80.2% vehicle vs. 78.3% naïve, $p < 0.01$) and again was unaffected by glibenclamide (I%BW = 80.5%). Contralateral (C) %BW in CCI+HS was increased in vehicle (78.6%) versus naive (78.3%, $p = 0.02$) but unchanged in CCI (78.3%). At 24 h, glibenclamide treatment in CCI+HS eliminated contralateral cerebral edema (C%BW = 78.3%) with no difference versus naïve. By 72 h, contralateral cerebral edema had resolved (C%BW = 78.5 $\pm$ 0.09% vehicle vs. 78.3 $\pm$ 0.05% naïve).

Glibenclamide decreased 24 h contralateral cerebral edema in CCI+HS. Contralateral edema did not develop in CCI alone. Surprisingly, 24 h of glibenclamide treatment failed to decrease ipsilateral edema in either model. These results may be related to glibenclamide dosing. Additionally, mechanisms underlying brain edema development may differ in injured versus contralateral hemispheres in CCI, with pericontusional edema (osmolar swelling) refractory to glibenclamide, but diffuse cerebral edema (via Sur1) produced by combined injury and/or resuscitation responsive to this therapy. TBI phenotype may mandate precision medicine approaches to treat brain edema.

Introduction

Background

3a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale

b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology

TBI) affects 1,700,000 people in the United States annually, with a mortality rate of 52,000. The prevalence of resultant disability is estimated at >5,000,000 people.[1,2] Secondary insults such as hypotension from polytrauma and hemorrhage are an important contribution to these unfavorable outcomes.[3,4] Hypotension is estimated to occur in >25% of severe TBI patients and can potentially double the mortality rate.[4] An analysis of 2061 patients with severe TBI and shock in the Resuscitation Outcomes Consortium trials revealed a mortality rate of 72% in patients with combined injury versus 46% in patients with severe TBI alone.[5] A critical factor believed to contribute to unfavorable outcome in these patients is the exacerbation of cerebral edema by secondary hypotension.[5] Hypotension is associated with diffuse cerebral edema, distinct from contusional swelling.[6] Although much emphasis has been placed on vasogenic edema in TBI at sites of injury/contusion (secondary to traumatic disruption of the blood–brain barrier), a growing body of evidence suggests the importance of diffuse cytotoxic edema in this disease triggered by factors such as global mitochondrial dysfunction, cell depolarization, ionic gradient alteration, and neurotransmitter release contributing to raised intracranial pressure (ICP).[7–11] Additionally, the large volumes of resuscitation fluids used to correct hypotension in these patients, although important to restoring an adequate cerebral perfusion pressure (CPP), exacerbate cerebral edema, thereby contributing to diffuse swelling and generating a vicious cycle. Given that cerebral edema is one of the most important pathophysiological factors associated with death and unfavorable outcomes in TBI, alternative therapeutic approaches are critically needed.[6,12–15]

Feinstein and coworkers suggested that resuscitation fluid requirements to restore CPP could be reduced with the use of pressor agents, and although this approach has some merit, it is largely unfeasible in the pre-hospital setting.[16] Few studies have been conducted targeting TBI resuscitation in patients with polytrauma.

Unfortunately, studies attempting to reduce resuscitation fluid volumes (with either albumin or hypertonic saline) have failed, or even resulted in worse outcomes than patients resuscitated with isotonic crystalloid.[17,18] Beyond vasopressors or small volume resuscitation solutions, other therapies targeting cerebral edema are reactionary. Even though osmolar agents, barbiturate coma, hypothermia, or decompressive craniectomy have clinical utility, they are morbid and associated with side effects and/or worsening of hemodynamic status, which may be highly problematic in the setting of polytrauma. Moreover, they are nonspecific and reactive rather than targeted and preventive of cerebral edema. They also have limited use in the pre-hospital setting. A pharmacological strategy given after resuscitation to prevent the progression of cerebral edema rather than treat it in a reactionary manner would be highly desirable to improve outcomes.

One potential strategy in this regard involves targeting a sulfonylurea receptor, Sur1.[19] Initially described for its central nervous system (CNS) effects in ischemic stroke, this pathway is now also being studied in TBI.[20,21] Sur1 is a transmembrane receptor that obligatorily associates with an adenosine triphosphate (ATP) and calcium-sensitive channel (transient receptor potential cation channel subfamily M member 4 [Trpm4]) and nonselectively conducts monovalent cations.[22,23] Injury and depletion of ATP causes upregulation of Sur1 and persistent activation of the Sur1–Trpm4 complex. This results in cell depolarization from sodium influx, causing intracellular edema and eventually cell death.[19,24] This pathway has been validated by persistent channel opening and development of edema without ATP depletion by diazoxide[24](which opens the channel), as well as reduction in oncotic cell death with channel blocking by glibenclamide.[19] Two major advantages of this pathway over other channels implicated in the process of cerebral edema are that Sur1-Trpm4 is not constitutively expressed in the CNS, but is selectively upregulated by injury, and it can be inhibited in humans by clinically available United States Food and Drug Administration [FDA]-approved medication for diabetes: glibenclamide, also known as glyburide.

Prior pre-clinical research on Sur1 and TBI[20] has focused on inhibiting Sur1 in pericontusional edema and hemorrhage rather than the diffuse cerebral edema that often causes marked elevation in ICP in TBI patients with secondary insults and resuscitation. Additionally, the role of the Sur1 pathway and the impact of agents targeting Sur1 in pre-clinical models of TBI with secondary insults such as hypotension from HS have not been examined, despite their potential importance.[3,25]

Objectives

4. Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested

To address these issues (described above) in evaluating the benefit of glibenclamide as an edema-prevention therapy, we used our established model of TBI plus HS in mice.[26–28] Also, because Sur1 requires upregulation, the effects on edema may not be apparent in the acute resuscitation period. We hypothesized that mice treated with glibenclamide would have reduced pericontusional and diffuse brain edema at 24 and 72 h after resuscitation. To better understand the contribution of the resuscitation to the development of brain edema after TBI and the impact of glibenclamide, we also studied the impact of glibenclamide therapy in TBI in the presence and absence of secondary HS.

Methods

Ethical statement

5. Indicate the nature of the ethical review permissions, relevant licenses (e.g., Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine and Operation Brain Trauma Therapy (approved protocol numbers 14013150, 17019898, and 17091175). Animals were handled in compliance with ARRIVE guidelines.

Study design

6. For each experiment, give brief details of the study design, including:

a. The number of experimental and control groups

Naïve: 10
24 h CCI vehicle: 10
24 h CCI glibenclamide: 10
24 h CCI + HS vehicle: 10 (1 excluded as outlier, 1 died)
24 h CCI + HS glibenclamide: 10
72 h CCI + HS vehicle: 9 (2 died)
72 h CCI + HS GLI: 9 (2 died)
72 h naïve: 10

b. Any steps taken to minimize the effects of subjective bias when allocating animals to treatment (e.g., randomization procedure) and when assessing results (e.g., if done, describe who was blinded and when)

Following glibenclamide treatment versus vehicle (see subsection below for dosing), mice were decapitated at 24 h. The brains were removed immediately and the hemispheres were bisected for quantification of brain edema by a technician blinded to treatment.

c. The experimental unit (e.g., a single animal, group, or cage of animals); a timeline diagram or flow chart can be useful to illustrate how complex study designs were implemented

Experiments were conducted in a group of mice (C57/BL6 male mice were used [Jackson Laboratories, Bar Harbor, ME), 12–15 weeks of age, weighing 25–30 g).

Flow chart included as Figure S1.

Experimental procedures

7. For each experiment and each experimental group, including controls, provide precise details of all procedures performed. For example:

a. How (e.g., drug formulation and dose, site and route of administration, anesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s).

This is described in the manuscript with specific sections following:

Injury by CCI or CCI+HS was induced in concordance with our standard established models that have been described previously and successfully used in our laboratory for prior investigations.[27–29] The level of injury in both CCI and CCI+HS was moderate at 5 m/sec

and 1 mm depth to the left parietal cortex. The craniotomies were sealed closed with Koldmount hardener immediately after impact.

CCI + HS. In the CCI+HS model, HS was induced per protocol by removing 2.3 mL blood/100 g over 15 min, followed by a controlled mean arterial pressure (MAP) of 25–27 mm Hg for 20 min maintained by removal or infusions of citrated autologous blood from the femoral venous catheter in 0.05 mL aliquots. This produced a 35 min period of severe hypotension. Following 35 min of HS, mice entered a ''pre-hospital'' phase for 90 min where they were resuscitated with lactated Ringer's (LR) solution for a MAP goal ≥70 mm Hg (initial bolus 20 mL/kg, and additional 10 mL/kg boluses over 5 min as needed to maintain MAP >70 mm Hg). The subsequent ''hospital'' phase involved reinfusion of the remaining shed blood over 15 min to mimic clinical care in emergency departments or combat hospitals.

Anesthesia. For all experiments, mice were anesthetized per our standard protocol with 4% isoflurane with a gas mixture of 2:1 nitrous oxide to oxygen. Isoflurane was reduced to 1–2% after surgical procedures and placed on room air 10 min prior to injury. Isoflurane was maintained at 1% with room air through the 90 min pre-hospital phase and then switched to 100% O_2 with 1% isoflurane through the hospital phase.

Euthanasia. Following glibenclamide treatment versus vehicle (see subsection on Glibenclamide treatment) mice were decapitated at 24 h. The brains were removed immediately and the hemispheres were bisected for quantification of brain edema by a technician blinded to treatment. The same protocol was followed to assess the effect of glibenclamide on cerebral edema at 72 h in the combined injury model CCI+HS ($n = 9$ per group).

Glibenclamide treatment. A stock solution of 2.5 μg/μl glibenclamide was made in 100% dimethyl sulfoxide (DMSO) from which a loading dose solution (20 μg/mL) was made in unbuffered normal saline. These solutions were used for all experiments. The effect of glibenclamide was assessed at 24 h ($n = 10$/group/model) and 72 h ($n = 9$/group/model) using an intravenous (IV) loading dose of glibenclamide (20 μg/kg) that was given 10 min post-CCI, or at the start of the pre-hospital phase in CCI+HS, followed by a continuous subcutaneous (SQ) infusion at 0.4 μg/h (Alzet mini-pump). This treatment regimen was derived as the mouse equivalent based on rat doses of 10 μg/kg used in prior studies of glibenclamide post-TBI and a body-surface-area-adjusted species conversion.[20,30,31] This protocol was used for all mice and continued for 24 h or 72 h after the insult, depending on the group being studied. In addition to studies assessing cerebrovascular and systemic hemodynamics after CCI+HS, we also used this protocol in separate mice to quantify glibenclamide levels in blood. Here, the infusion was continued for 4 days.

Glibenclamide level determination. Glibenclamide levels ($n = 5$/group) were determined by Ultra Performance Liquid Chromatography (UPLC)-mass spectrometer (MS) MS/MS 15 min post-IV load, 1 h post loading dose + SQ pump infusion, and at 4 days post loading dose + SQ pump infusion to determine steady state levels of the drug. A separate cohort of uninjured mice were used for this determination, because the volume of plasma required by the UPLC MS/MS method were prohibitive in terms of increasing mortality as well as altering our established model. Repeated withdrawal of blood samples in a model of HS would potentially also alter the fluid resuscitation strategy after HS and further influence edema. Glibenclamide levels were compared with vehicle ($n = 3$). The UPLC-MS/MS method[32] involved liquid-liquid extraction and detection with a triple quadrupole mass spectrometer. Serum (0.2 mL), spiked with glimiperide as internal standard was acidified with hydrochloric acid and extracted with hexanes:methylene chloride (50:50), dried under a gentle stream of nitrogen, and reconstituted in 50 μL of 50:50 acetonitrile:deionized water. Glibenclamide and glimiperide were eluted from a Waters Acquity UPLC BEH C18, 1.7 μm, 2.1 × 150 mm reversed-phase column, isocratically with acetonitrile: water (0.1% formic acid) 50:50. Detection and quantitation were achieved in the positive mode with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via a heated electrospray ionization (HESI) probe with the Waters UPLC Acquity solvent delivery system. Transitions used for analysis were 494.1 → 368.9 for glibenclamide and 491 → 352 for the internal standard. The calibration curves, obtained from extracting known concentrations of glibenclamide from double-stripped serum, ranged from 0.1 ng/mL (lower limit of quantitation) to 16 ng/mL. All back calculations of calibrators, inter-day and intra-day precision and accuracy, and stability were within acceptable limits. Concurrent glucose levels for all animals at baseline and the abovementioned time points after HS at 35min, PH at 2h and HOSP at ∼2.5h were obtained using a blood gas analyzer (Model ABL-90, Radiometer America, Westlake, OH).

Determination of brain edema. %BW was quantified for all mice using the established wet-dry weight technique, which represents a gold standard for its assessment.[33] Because perfusion with normal saline alters water levels and provides inaccurate assessment of edema, mice were not perfused with normal saline before harvesting brains for water measurements. Rather, at the completion of the injury described previously, mice were decapitated at each time point (under 5% isoflurane and 50/50 gas mixture of nitrous oxide and oxygen) and the brain was bisected into hemispheres that were immediately weighed; these weights were recorded as wet weights. Hemispheres were subsequently dehydrated for 72 h in an oven at 110°C and re-weighed to record dry weights. %BW was determined by subtracting the dry from the wet weight, dividing this number by the wet weight, and multiplying by 100.

b. When (e.g., time of day)

Procedures were performed during daylight hours.

c. Where (e.g., home cage, laboratory, water maze)

Surgeries and determination of %BW were performed in the laboratory

d. Why (e.g., rationale for choice of specific anasthetic, route of administration, drug dose used)

Rationales for anesthetic, drug dose used are provided in previous sections.

Experimental animals

8a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g., mean or median age plus age range), and weight (e.g., mean or median weight plus weight range)

Experiments were performed in a group of mice (C57/BL6 male mice were used (Jackson Laboratories, Bar Harbor, ME), 12–15 weeks of age, weighing 25–30 g).

b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g., knockout or transgenic), genotype, health/immune status, drug- or test-naıve, previous procedures, etc.

NA

Housing and husbandry

9. Provide details of:

a. Housing (e.g., type of facility, e.g., specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish).

All mice for these experiments were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved university managed animal facility. The facility is a SPF-free facility. Standard mouse box with HIPAA approved filtered lids were utilized in individually ventilated cages (IVC). Standard approved rodent bedding was used in each cage. Mice for these experiments were exempt from the social housing protocol; therefore, mice were single housed for the duration of each experiment.

b. Husbandry conditions (e.g., breeding program, light/dark cycle, temperature, quality of water etc. for fish, type of food, access to food and water, environmental enrichment)

Housing facility was maintained at 20–22.2°C, with a 12 h light/dark cycle. Animals were allowed 24 h/day 7 day/week access to food and water. Tap water and standard rodent chow were provided to all mice. These mice were exempt from university environmental enrichment program and were housed with only a nestlet.

c. Welfare-related assessments and interventions that were conducted before, during, or after the experiment

Animals' welfare was maintained according to the Guide and Use for Laboratory Animals requirements for the entirety of the experiments.

Sample size

10a. Specify the total number of animals used in each experiment and the number of animals in each experimental group

Naïve: 10
24 h CCI vehicle: 10
24 h CCI glibenclamide: 10
24 h CCI + HS vehicle: 10 (1 excluded as outlier, 1 died)
24 h CCI + HS glibenclamide: 10
72 h CCI + HS vehicle: 9 (2 died)
72 h CCI + HS glibenclamide: 9 (2 died)
72 h naïve: 10

b. Explain how the number of animals was decided; provide details of any sample size calculation used

Effect sizes of glibenclamide on brain water were unknown; therefore, sample sizes were determined empirically as 10/group.

c. Indicate the number of independent replications of each experiment, if relevant

Each experiment was performed independently in 10 animals per group (see *a*).

Allocating animals to experimental groups

11a. Give full details of how animals were allocated to experimental groups, including randomization or matching if done

Animals were randomized after injury to either the treatment group or vehicle group. Because of the length of these studies, a block randomization was used each week to ensure that some animals were added to either the treatment group or the vehicle group each week.

b. Describe the order in which the animals in the different experimental groups were treated and assessed

A trained senior technician treated and assessed each animal in each experimental group. All portions of the study were done blinded so as not to introduce bias.

Experimental outcomes

12. Clearly define the primary and secondary experimental outcomes assessed (e.g., cell death, molecular markers, behavioral changes)

The primary outcome assessed was brain edema as determined by %BW

Details from manuscript follow

%BW was quantified for all mice using the established wet-dry weight technique, which represents a gold standard for its assessment.[33] Because perfusion with normal saline alters water levels and provides inaccurate assessment of edema, mice were not perfused with normal saline before brains were harvested for water measurements. Rather, at the completion of the injury described previously, mice were decapitated at each time point (under 5% isoflurane and 50/50 gas mixture of nitrous oxide and oxygen) and the brain was bisected into hemispheres that were immediately weighed; these weights were recorded as wet weights. Hemispheres were subsequently dehydrated for 72 h in an oven at 110°C and re-weighed to record dry weights. %BW was determined by subtracting the dry from the wet weight, dividing this number by the wet weight, and multiplying by 100.

Statistical methods

13a. Provide details of the statistical methods used for each analysis.

b. Specify the unit of analysis for each data set (e.g., single animal, group of animals, single neuron)

c. Describe any methods used to assess whether the data met the assumptions of the statistical approach

Described in manuscript

Glibenclamide levels, glucose concentrations, and ipsilateral and contralateral %BW were reported as means ± standard error. Normality was determined by Q-Q plots (Fig. S2). Differences between %BW in naive, vehicle-, and glibenclamide-treated animals were assessed using one way ANOVA, and between-group comparisons were made using Student's *t* test. Multiple comparisons during post-hoc analyses were adjusted for using Bonferroni's correction. Physiological parameters were analyzed by repeated measures ANOVA. Outliers were excluded using Dixon's test. Statistical significance was determined by p values <0.05. All statistical tests were performed using Stata 13.1 (StataCorp, College Station, TX).

Results

Baseline data

14. For each experimental group, report relevant characteristics and health status of animals (e.g., weight, microbiological status, and drug- or test-naıve) before treatment or testing (this information can often be tabulated)

All animals analyzed were in good health prior to injury and were naïve to the drug before treatment/testing.

Numbers analyzed

15 a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g., 10/20, not 50%).

b. If any animals or data were not included in the analysis, explain why.

Naïve: 10 analyzed
24 h CCI vehicle: 10 analyzed
24 h CCI glibenclamide: 10 analyzed
24 h CCI + HS vehicle: 8 analyzed (1 excluded as outlier, 1 died)
24 h CCI + HS glibenclamide: 10 analyzed
72 h CCI + HS vehicle: 7 analyzed (2 died)
72 h CCI + HS glibenclamide: 7 analyzed (2 died)
72 h naïve: 10 analyzed

Outcomes and estimation

16. Report the results for each analysis performed, with a measure of precision (e.g., standard error or confidence interval)

Reported in manuscript (see subsequent text)

Steady state glibenclamide levels do not decrease glucose levels in mice

Previous studies in rats suggest that a loading dose of 10 μg/kg intraperitoneal (IP) followed by 200 ng/h of glibenclamide SQ infusion yields a plasma concentration of $\sim$5 ng/mL (Simard, unpublished observation) and does not affect serum glucose.[19,34,35] Our protocol estimated the effects of an equivalent dose in mice; the 15 min post-IV load levels of glibenclamide were 6.26 ± 3.55 ng/mL and the 4 day steady state levels were 7.72 ± 1.23 ng/mL (Fig. 1A). As expected, levels were undetectable in naïve mice. At these levels, serum glucose remained normal, was not significantly different than baseline, and there were no episodes of hypoglycemia in any individual mouse (Fig. 1B).

Glibenclamide treatment does not affect ipsilateral edema, but decreases contralateral edema at 24 h after combined injury

In the contused hemisphere (ipsilateral to TBI), edema was increased at 24 h after CCI+HS (ipsilateral %BW = 80.46 ± 0.14% vehicle [n = 8] vs. 78.31 ± 0.04% naïve [n = 10], $p < 0.001$) but surprisingly not reduced by glibenclamide (ipsilateral %BW = 80.42 ± 0.24%, p = 1.0, n = 10, power = 1.0 Fig. 2A-i). Edema in the hemisphere ipsilateral to TBI also developed in CCI alone (ipsilateral %BW = 80.20 ± 0.15 vehicle [n = 10] vs. 78.31 ± 0.04% naïve (n = 10) $p < 0.001$) and again was not attenuated by treatment with glibenclamide (ipsilateral %BW = 80.5 ± 0.13% [n = 10], p = 0.18, power = 1.0, Fig. 2A-ii).

We also detected cerebral edema in the hemisphere contralateral to injury in the CCI+HS model, but not in CCI alone (Fig. 2B). Contralateral %BW in the combined injury of CCI+HS was increased in vehicle (78.65 ± 0.10%, n = 8) versus naive (78.24 ± 0.05%, n = 10, p = 0.014) but unchanged versus naïve in CCI alone (78.28 ± 0.08%, n = 10, p = 1.0). However, in contrast to what was observed in the hemisphere ipsilateral to injury, at 24 h, glibenclamide treatment after CCI+HS eliminated brain edema in the contralateral hemisphere versus vehicle (contralateral %BW = 78.25 ± 0.10%, n = 10, p = 0.011; power = 0.904) returning %BW levels to naïve levels (Fig. 2B-ii). It is of note that one of the vehicle-treated mice in CCI+HS died before 24 h. There were no deaths in the glibenclamide-treated group. One CCI+HS vehicle outlier was excluded. There was no difference in physiological parameters that could influence cerebral edema/intracranial pressure including MAP, sodium levels, or serum osmolarity between the vehicle and glibenclamide groups (Fig. 4 A-i, B-i, C-i).

Glibenclamide treatment does not affect 72 h ipsilateral edema in combined injury

Ipsilateral edema remained increased at 72 h in CCI+HS (ipsilateral %BW = 80.37 ± 0.04% vehicle vs. 78.31 ± 0.04% naïve, $p < 0.001$) but again was not reduced by glibenclamide (p = 1.0, power = 1.0, Fig. 4A). Contralateral edema largely resolved in the combined injury model by 72 h (contralateral %BW = 78.45 ± 0.09% vehicle vs. 78.26 ± 0.05% naïve, p = 0.24, power = 0.46 Fig. 4B). Two animals in both vehicle- (n = 9) and glibenclamide- (n = 9) treated groups died by 72 h after injury, consistent with the observed level of mortality in our prior reports with this severe combined injury model.[20] MAP, serum sodium levels and serum osmolarity were not different between the treatment and vehicle groups (Fig. 3 A-ii, B-ii, C-ii).

Adverse events

17a. Give details of all important adverse events in each experimental group

In 72h CCI+HS (vehicle and glibenclamide groups), two animals died, and in 24 h CCI+HS (vehicle group), one animal died.

b. Describe any modifications to the experimental protocols made to reduce adverse events

This mortality rate is consistent with the observed level of mortality in our prior reports with this severe combined injury model.[20]

Discussion

Interpretation/scientific implications

18a. Interpret the results, taking into account the study objectives and hypotheses, current theory, and other relevant studies in the literature

b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results

c. Describe any implications of your experimental methods or findings for the replacement, refinement, or reduction (the 3Rs) of the use of animals in research

These points are addressed in the manuscript Discussion section. Specific sections follow.

Our study demonstrates the following findings:(1) a combined injury model of TBI (by CCI) and hypotension (by HS) followed by resuscitation with LR generates a significant amount of edema in the contused hemisphere, and also results in diffuse edema in the contralateral hemisphere; (2) in this injury model and treatment regimen, glibenclamide decreases (or prevents generation of) edema in the contralateral hemisphere back to baseline by 24 h; (3) contralateral cerebral edema is largely resolved by 72 h; (4) in mice with isolated CCI, edema is restricted to the hemisphere ipsilateral to the impact, and (5) surprisingly, in both models, glibenclamide treatment failed to attenuate edema in the contused hemisphere ipsilateral to impact.

Ipsilateral edema is not responsive to this dose of glibenclamide therapy in mice

The failure of glibenclamide to attenuate edema in the contused hemisphere in our study, although unexpected, supports the growing body of literature demonstrating that there are multiple mechanisms underpinning different types of cerebral edema generated in TBI and that these mechanisms may require unique and targeted therapies and specific doses/durations of therapy. It is possible, indeed likely, that the Sur1/glibenclamide pathway is one of many upregulated edema-generating mechanisms, and the effects of its inhibition may, therefore, be diluted/overwhelmed by other mechanisms. These mechanisms may include a profound osmolar gradient caused by the contusion resulting in edema refractory to Sur1 targeted therapy even though Sur1 upregulation has been demonstrated in the contusion/pericontusion.[20,30,38,39] It is also possible that glibenclamide treatment may influence additional targets other than Sur1 related to cerebral edema and/or neuroprotection.[40]

Diffuse cerebral edema after combined injury is responsive to this dose of glibenclamide therapy at 24 h

Earlier theories of the etiology of edema in TBI being caused by vascular engorgement have now been replaced with evidence that there is usually a key cytotoxic component, particularly to perilesional and diffuse edema.[8,10,53–57] In addition, some component of vasogenic edema thought to be secondary to blood–brain barrier (BBB) compromise may also contribute to diffuse edema.[10,58,59] BBB permeability to albumin is not seen in the contralateral hemisphere in CCI alone.[60] The Sur1 pathway has been implicated in its contributions to both cytotoxic and vasogenic components of brain edema: association with Trpm4 in neurons causes rapid cell depolarization, influx of sodium, followed by intracellular (i.e., cytotoxic) edema and eventually oncotic death. The same process in CNS vascular endothelial cells results in degradation of the tight junctions and compromises the BBB, allowing extravasation of proteinaceous fluid (i.e., vasogenic edema).[61] Prior studies evaluating the effect of glibenclamide in TBI have used standard models of focal cortical contusion and predominantly focused on the ipsilateral hemisphere, hippocampal injury, and impact on parenchymal hemorrhage.[20,21] Although increased Sur1 expression has been detected in the contralateral hemisphere after injury,[20] its role, particularly as it relates to diffuse edema, has been characterized to a lesser extent. This is likely because models of CCI alone, at various injury levels, typically may not produce a significant amount of diffuse brain edema, as demonstrated by our study and other reports, when water content in contralateral hemispheres is tested separately from hemispheres ipsilateral to injury.[8,31,62–71]

Glibenclamide in CCI

There have been four published pre-clinical studies evaluating glibenclamide specifically in TBI.[20,30,31,40] These studies examine CCI alone, and none evaluate glibenclamide in a combined model of CCI plus a secondary insult such as HS. In two of these studies (using 10 μg/kg glibenclamide in rats) reduced progressive secondary hemorrhage and improved behavioral outcomes were noted; however, cerebral edema was not assessed.[20,30] Edema was evaluated by Zweckberger and coworkers in a rat model of CCI: 10 μg/kg of glibenclamide treatment did not affect acute intracranial pressure but decreased 24 h ipsilateral BW and contusion volume (at 8 h, 24 h, 72 h, and 7 days).[32] The most recent study by Xu and coworkers,[40] also evaluated isolated CCI injury and brain edema in mice. They reported reduction in ipsilateral brain water (at day 3 post-TBI) and BBB disruption after 10 μg intraperitoneal injection of glibenclamide for 3 days, and implicated the role of an alternative pathway (c-Jun N-terminal kinase [JNK]/c-jun mediated apoptosis). Although these reports confirm our finding of no contralateral edema development in rats or mice after CCI alone, contrary to our results, both reported reduction in ipsilateral brain water at 24 h.

There are multiple essential differences between these reports and our study including (1) methodologies to determine brain edema, (2) glibenclamide treatment dose, (3) treatment duration, (4) species, and (5) injury severity. Although we used body-surface-area-adjusted dose conversions to achieve the murine equivalent of 10 μg/kg in rats, and with this regimen achieved similar glibenclamide levels compared with unpublished observations by Simard and coworkers,[35] other reports of glibenclamide in murine models of non-TBI disease use higher doses (10 μg/mouse) with reduced *in vivo* neuronal damage, preservation of myelin, preservations of axons, and more numerous/mature oligodendrocytes and reduced *in vitro* glutamate-induced cell swelling in experimental autoimmune encephalitis.[83,84] This, combined with the results from Xu and coworkers, indicate that differences in murine dosing may be an important contributor to the variation in results observed between our study and prior reports.

The varied results of these pre-clinical studies highlight the complexity and heterogeneity of TBI, and, therefore, the importance of studying different doses, treatment durations, models, species, and injuries so that pre-clinical models can more accurately inform clinical studies. Indeed, a recent small randomized controlled trial in moderate-severe TBI (diffuse axonal injury) suggests that glibenclamide may be neuroprotective. The mechanism of neuroprotection is currently unclear and may include pathways distinct from cerebral edema.[85] Although negative murine studies may not be directly predictive of results in humans, they can inform optimal treatment regimens and clinical trial design.

Generalizability/translation

19. Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology

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Funding

20. List all funding sources (including grant numbers) and the role of the funder(s) in the study

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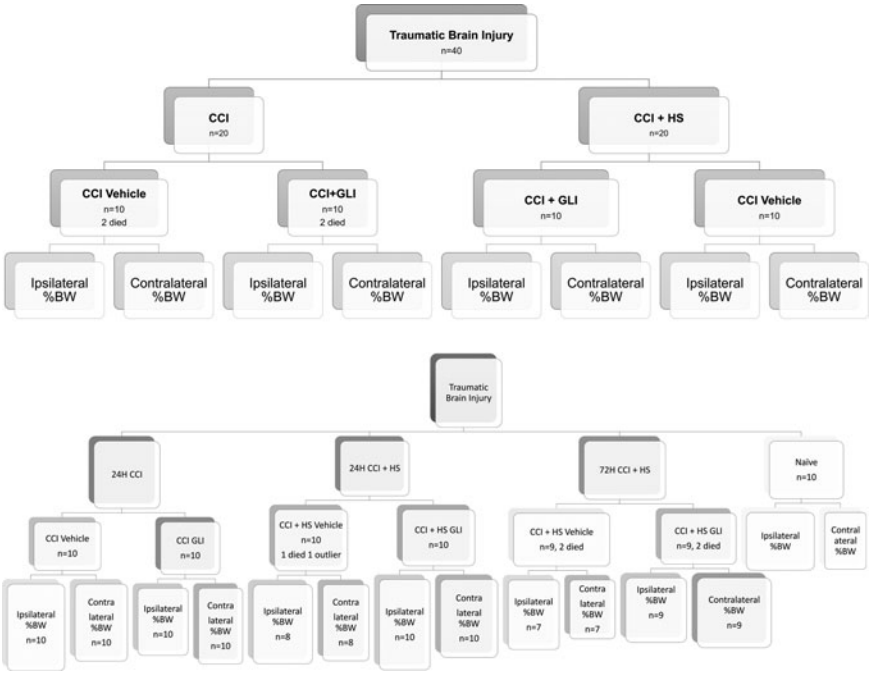
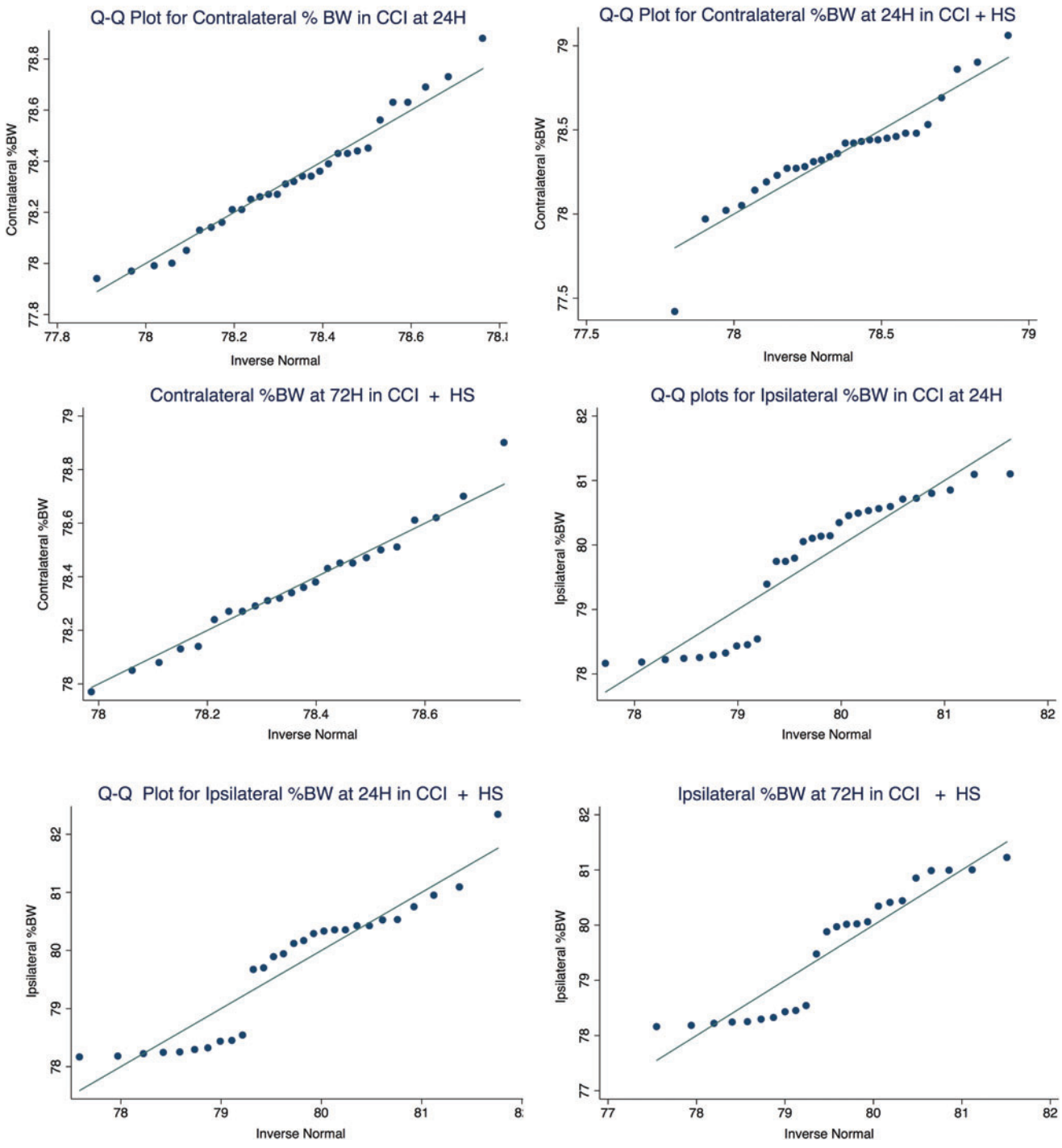
These resources have funded materials, animals, and technician and investigator salary support.



SUPPLEMENTARY FIG. 1. Schematic of experiments determining number of animals injured with controlled cortical impact (CCI) alone (with vehicle versus glibenclamide, GLI) compared with mice subjected to CCI + hemorrhagic shock (HS), again treated with vehicle or GLI. In each case, ipsilateral and contralateral percent brain water (% BW) was measured.



SUPPLEMENTARY FIG. S2. Q-Q plots. Top left: Q-Q plot for contralateral % brain water (BW) in controlled cortical impact (CCI) at 24 h. Top right: Q-Q plot for %BW at 24 h in CCI + hemorrhagic shock (HS). Center left: contralateral %BW at 72 h in CCI+HS. Center right: Q-Q plots for ipsilateral %BW in CCI at 24 h. Bottom left: Q-Q plot for ipslilateral %BW at 24 h in CCI+HS. Bottom right: ipsilateral %BW at 72 h in CCI+HS.