

# Hypertonic Resuscitation Improves Neuronal and Behavioral Outcomes after Traumatic Brain Injury plus Hemorrhage

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**Background:** Resuscitation with hypertonic saline or hypertonic saline plus L-arginine acutely improves cerebral blood flow after traumatic brain injury (TBI) followed by hemorrhagic hypotension. The authors investigated whether hypertonic saline or hypertonic L-arginine would improve long-term neuronal survival and behavioral outcomes 15 days after TBI and hemorrhagic hypotension.

**Methods:** Mean arterial pressure, arterial blood gases, pH, plasma glucose, hematocrit, and hemoglobin were measured in male Sprague-Dawley rats before and after moderate (2.0 atm) fluid percussion TBI. Rats were assigned to one of six groups: (1) sham TBI, (2) hemorrhage only, (3) TBI only, (4) TBI plus hemorrhage and resuscitation with 0.9% saline, (5) TBI plus hemorrhage and resuscitation with hypertonic saline (7.5%), or (6) TBI plus hemorrhage and resuscitation with L-arginine (100 mg/kg) in hypertonic saline. On postinjury days 1–5, vestibulo-motor function was assessed using beam balance and beam walking tasks. On postinjury days 11–15, spatial memory function was assessed using the Morris water maze. After behavioral testing, neuronal counting was performed bilaterally on specific hippocampal regions.

**Results:** Groups receiving hypertonic saline ( $P < 0.05$ , day 15 vs. day 11) or hypertonic L-arginine ( $P < 0.05$ , days 13–15 vs. day 11) showed improved performance over time on the Morris water maze, as well as significantly improved neuronal survival in the contralateral hippocampus ( $P < 0.05$ , hypertonic saline or hypertonic L-arginine vs. normal saline) compared with untreated TBI or normal saline-treated TBI plus hemorrhage groups.

**Conclusions:** Hypertonic saline and hypertonic L-arginine were both effective at promoting long-term neuronal survival and behavioral recovery. The slightly earlier improvement in Morris water maze performance in the hypertonic L-arginine group warrants further studies to determine whether higher doses of L-arginine provide additional improvement. This study supports the therapeutic benefits of hypertonic resuscitation after TBI plus hemorrhagic hypotension.

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PATIENTS with traumatic brain injury (TBI) often present with hypoxia and hypotension that can lead to secondary ischemic injury.<sup>1</sup> Studies of brain-injured patients indicate that although both hypoxia and hypotension increase morbidity and mortality, it is hypotension, independent of hypoxia, which is the major contributor to secondary ischemic damage. Hypotension that occurs any time between onset of the injury and resuscitation treatment is more likely to contribute to long-term cognitive deficits and mortality than hypoxia alone.<sup>2,3</sup> Severe head injury is associated with impaired cerebral blood pressure autoregulation; the combination of lost autoregulation and systemic hypotension leads to cerebral hypoperfusion and probably to secondary ischemic damage (for review, see Golding *et al.*,<sup>4</sup> 1999; DeWitt and Prough,<sup>5</sup> 2003).

Although other factors such as seizures and hyperthermia also may contribute to the development of secondary cerebral ischemia after TBI, it seems that systemic arterial hypotension is the primary contributor. Patients with posttraumatic hypotension are three times more likely to die when compared with those who have normal blood pressure.<sup>3</sup> Therefore, a therapeutic intervention to prevent or treat posttraumatic hypotension and prevent secondary ischemia would be a powerful tool in improving outcome after brain injury.

Current treatments of posttraumatic hypotension include the use of resuscitation fluids or pharmacologically active agents with small volumes of fluids to restore systemic blood pressure. Various resuscitation fluids have been used with varying degrees of success; examples include lactated Ringer's solution, 0.9% saline, mannitol, hypertonic saline, and hypertonic saline with dextran.<sup>6</sup> Hypertonic saline solutions, with or without added colloid, effectively increased blood pressure and reduce intracranial pressure (ICP) in TBI patients with intracranial hypertension.<sup>7</sup> Furthermore, patients resuscitated with hypertonic saline had higher systolic blood pressures and enhanced survival compared with those who received a 0.9% saline bolus.<sup>8</sup> Brain-injured patients with hypotension who received hypertonic saline with dextran were twice as likely to survive as those who received standard care.<sup>9</sup>

L-Arginine, a precursor to the potent vasodilator nitric oxide, increases blood flow to vital organs,<sup>10</sup> possibly through vasodilator actions that contribute to maintaining nutritional flow to tissue during acute hemorrhage.<sup>11</sup> In hemorrhaged rats, L-arginine improved survival rate<sup>12</sup>

and macrophage function.<sup>13</sup> Under normal physiologic conditions, L-arginine concentrations in endothelial cells are sufficient for basal production of nitric oxide (*i.e.*, L-arginine availability is not rate limiting); however, under conditions of excessive ischemia-induced formation of nitric oxide, local L-arginine concentrations may be inadequate.<sup>14</sup> Therefore, treatment with exogenous L-arginine during the early posthemorrhagic period may contribute to improved cerebral blood flow (CBF) after TBI and hemorrhagic hypotension.

When administered after experimental TBI, L-arginine prevented posttraumatic hypoperfusion and increased tissue nitric oxide levels.<sup>15-17</sup> Furthermore, when rats were subjected to hemorrhagic hypotension (mean arterial pressure [MAP] 60 mmHg for 45 min) immediately after TBI, hypertonic L-arginine (2,400 mOsm/kg) and hypertonic saline (2,400 mOsm) prolonged the duration of increased CBF for up to 2 h after injury without increasing ICP.<sup>18</sup> In contrast, in a rodent model of hemorrhagic shock (35–40 mmHg for 30 min), L-arginine increased susceptibility to organ damage and reduced survival time,<sup>19</sup> suggesting that hypertonic L-arginine may contribute to acute improvements in CBF without improving long-term outcome. Therefore, the current study was designed to investigate whether the acute restoration of CBF with hypertonic resuscitation would be associated with long-term improvement in behavioral or neuropathologic outcome after TBI and hemorrhagic hypotension in rats.

## Materials and Methods

### Animals

Adult (3–4 months) male Sprague-Dawley rats (350–400 g) were obtained from Harlan (Houston, TX), housed two per cage with food and water *ad libitum*, and maintained at a constant temperature (21°–23°C) and humidity (45–50%) with lights on 07:00–19:00 h. After surgical manipulations, animals were housed singly. Behavioral measures were conducted during the light part of the light–dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas.

### Surgical Preparation

Rats were anesthetized with 4% isoflurane in an anesthetic chamber for 5 min, intubated, and mechanically ventilated with 1.5–2% isoflurane in oxygen: room air (50:50) using a volume ventilator (EDCO Scientific, Chapel Hill, NC) set at a tidal volume of 12–15 ml/kg and 40 breaths/min. Polyethylene cannulae were placed in

the right common jugular vein (drug infusion, hemorrhage, and blood sampling) and the tail artery (arterial pressure monitoring). Rectal and temporalis muscle temperatures were monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH), and rectal temperature was maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Body temperatures were maintained between 36.4° and 37.7°C for the duration of anesthesia.

Rats were prepared for paramedian fluid percussion TBI as previously described.<sup>15,20</sup> Briefly, rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.0-mm-diameter hole was trephined into the skull 2.0 mm to the right of the sagittal suture and midway between lambda and bregma. A modified Luer-Lok syringe hub (Becton-Dickinson, Franklin Lakes, NJ) was placed over the exposed dura, bonded in place with cyanoacrylic adhesive, and covered with dental acrylic. Isoflurane was decreased to 1.5%; the rats were connected to the trauma device and subjected to moderate (2.0 atm) fluid percussion TBI. Immediately after TBI, in rats that were receiving hemorrhage, MAP was reduced to 60 mmHg for 45 min by removing blood from the internal jugular vein. Shed blood was discarded. After 45 min, rats received their resuscitation treatments (see below) infused over 6 min. Blood samples were collected at 60 and 90 min after TBI in all groups. Rats were monitored for 90 min after TBI and given 120 mg/kg acetaminophen before arousal. Wound sites were treated with a topical antibiotic and infused with a local anesthetic, and the rats were allowed to recover in a warm, humidified incubator.

Rats were randomly assigned to one of six groups: (1) sham TBI, surgical preparation without TBI (SHAM; *n* = 5); (2) hemorrhage only, arterial blood withdrawn to lower MAP to 60 mmHg for 45 min followed by resuscitation treatment with 0.9% saline (1 ml/kg intravenously over 6 min; HEM; *n* = 8); (3) TBI, moderate fluid percussion injury without treatment (*n* = 5); (4) TBI followed by hemorrhage and resuscitation with 0.9% saline (1 ml/kg intravenously over 6 min; THS; *n* = 8); (5) TBI followed by hemorrhage and resuscitation with hypertonic saline (7.5% saline 1 ml/kg over 6 min; THH; *n* = 8); or (6) TBI followed by hemorrhage and resuscitation with hypertonic L-arginine (100 mg/kg in 1 ml/kg hypertonic saline over 6 min; THA; *n* = 9).

### Behavioral Assessment

Vestibulomotor function was assessed on postinjury days (PIDs) 1–5 using beam balance and beam walking tasks.<sup>20–22</sup> Spatial learning and memory were assessed on PIDs 11–15 using the Morris water maze.<sup>23–25</sup>

**Beam Balance.** The balance beam apparatus consisted of a beam 60 cm in length × 1.75 cm in width × 4.0 cm in height elevated 90 cm off the floor, and a barrier 30 cm in height × 30 cm in width.<sup>20,26</sup> The beam was secured to a table, and the barrier was attached to

the beam so that 50 cm of the beam protruded from the barrier, away from the table, over a cushioned safety box.

Rats were trained 24 h before injury, and one preassessment was made before injury. For training, each rat was placed on the balance beam for a 60-s trial. The rat was removed from the beam for a 15-s resting period between each trial. If the rat was not able to balance on its own, it was allowed to fall from the beam into the safety box. Timing began when the rat was securely positioned on the beam. The rat was considered trained when it was able to remain on the beam for three consecutive 60-s trials. Rats were further assessed (three consecutive, 60-s trials) on the day of injury before surgery and PIDs 1–5. Each trial was scored as follows: 1 = balances with steady posture (grooms, climbs barrier); 2 = grasps sides of beam and/or has shaky movements; 3 = hugs beam or slips or spins on beam; 4 = attempts to balance, but falls off after 10 s; 5 = drapes over beam or hangs from beam and falls off in less than 10 s; or 6 = falls off, makes no attempt to balance or hang from beam. Hence, a lower score indicates better performance.

**Beam Walk.** The beam walk apparatus consisted of a beam 100 cm in length  $\times$  2.5 cm in width  $\times$  4.0 cm in height with four equally spaced pegs (2 cm in height, 25 cm apart) and a black goal box (28 cm in length  $\times$  18 cm in height  $\times$  18 cm in width) at one end.<sup>20,27</sup> The starting end was stabilized by a stand, and the target end was attached to the goal box that rested on a table. The beam was elevated 1 m off the floor, and a bright light and white noise source were positioned near the starting end.

Each rat was trained 24 h before injury. First the rat was placed in the goal box for 2 min. At the end of 2 min, the rat was removed from the goal box, the light and white noise were turned on, and the rat was placed on the beam at the location of the peg hole closest to the goal box and allowed to walk to the goal box. As soon as the rat's front feet crossed the threshold of the goal box, the light and noise source were turned off. The animal was allowed to rest in the goal box for 30 s between each trial. This procedure was repeated twice at each peg location and from the starting position. The pegs were then inserted, and one complete beam walk was done for practice. Three timed beam walk trials were then recorded to conclude training. On the day of injury, the animal underwent a preassessment consisting of three timed trials. Three trial assessments were performed on PIDs 1–5.

**Morris Water Maze.** The water maze consisted of a 1.8-m-diameter tank, filled to 2 cm above an invisible platform, 10 cm in diameter and 26 cm high.<sup>22,23,25</sup> The water temperature was held at 24°–25°C. The platform was stationary throughout the experiment. Each rat's starting point was randomly assigned each day based on

four quadrants; one trial was started from each quadrant each day. The SMART computer system (San Diego Instruments, San Diego, CA) was used for tracking. The animal was allowed 2 min to locate and climb onto the platform and then 30 s to remain on the platform. If the rat did not locate the platform, it was placed on it for 30 s before removing it from the tank. Animals were given a 4-min rest period in a warming chamber between each trial.

#### *Neuronal Counting*

After the last water maze trial (PID 15), rats were anesthetized and perfused transcardially with saline followed by 10% formalin. Brains were removed, dehydrated in graded alcohols, and embedded in paraffin. Coronal sections (5–7  $\mu$ m, 100 sections per brain) were cut from the trauma site ( $-4$  mm from bregma), and every 20th section was stained with hematoxylin and eosin. Neuronal counting was performed bilaterally on stitched multiple images of the hippocampal areas CA1 (1.5-mm-long segment proximal to CA2), CA2–3, and the hilus of the dentate gyrus. High resolution digitized images of the hippocampal areas of interest were obtained by automatically acquiring multiple fields using a 10 $\times$  0.3 numerical aperture lens in an inverted Nikon TE200 microscope equipped with a Photometrics CoolSnap HQ digital camera (Roper Scientific, Tucson, AZ) and a Xenon arc lamp illuminator (Sutter Instrument Company, Novato, CA) plus motorized emission filter wheel and XY translation stage (Prior Scientific Inc., Rockland, MA). Each field was composed by a transmission and a fluorescence (490 nm excitation, 525 nm emission) image, which were combined during automatic image analysis for contrast enhancement as an aid for neuronal identification. Instrumentation control was done using Metamorph version 6.1 Software (Molecular Devices Corp., Downingtown, PA). Image stitching was performed using Metamorph in combination with the "photomerge" function of Adobe Photoshop Elements (Adobe Systems Inc., San Jose, CA). Automatic neuronal counting was performed by combining multiple morphometry segmentation and image analysis functions of Metamorph Software using custom-made journals (macro language). The automatic analysis was optimized by comparing it with manual counting in at least 10 preparations. All sections and images were coded to blind neuronal counting.

#### *Drugs*

Drugs included hypertonic saline (7.5%, 2,400 mOsm), and L-arginine (Sigma, St. Louis, MO) in hypertonic saline solution with sufficient sodium chloride added to produce total osmolality of 2,400 mOsm/kg.

#### *Data Analysis*

**Behavioral Data.** For the beam balance data, each day was summarized as behavior level 1 (all scores = 1)

**Table 1. Mean Arterial Blood Pressure**

Group	0 min	5 min	45 min	55 min	60 min	90 min
SHAM	92.7 ± 6.2	86.4 ± 11.1	82.3 ± 3.7	82.2 ± 5.8	84 ± 6.6	86.8 ± 6.3
HEM	95.2 ± 9.6	61.4 ± 1.2	62.3 ± 1.7	80.8 ± 12.6	83.1 ± 14.1	83.5 ± 11.1
TBI	91.7 ± 6.9	79.3 ± 15.3	81.6 ± 11	82.9 ± 10.5	82.7 ± 10.6	90.9 ± 7.0
THS	96.8 ± 10.7	64.3 ± 11	62.1 ± 3.5	84 ± 15.2	83.9 ± 16	78.9 ± 13.2
THH	93.5 ± 7.4	62.1 ± 2.3	60.6 ± 2.5	94.3 ± 12.9	95.2 ± 13.6	88.3 ± 8.9
THA	92.8 ± 5.3	64.9 ± 7.7	62.5 ± 4.8	90.6 ± 7.8	90.7 ± 9.4	82.1 ± 16.3

Mean arterial blood pressure in rats with (TBI) or without (SHAM) moderate fluid percussion traumatic brain injury, hemorrhagic hypotension (HEM; 60 mm Hg for 45 min), and resuscitation with saline (THS), hypertonic saline (THH), or L-arginine (100 mg/kg) in hypertonic saline (THA). See text for statistical analysis.

HEM = hemorrhage and resuscitation with normal saline; SHAM = sham surgery only; TBI = traumatic brain injury only; THA = TBI plus hemorrhage and resuscitation with L-arginine in hypertonic saline; THH = TBI plus hemorrhage and resuscitation with hypertonic saline; THS = TBI plus hemorrhage and resuscitation with normal saline.

versus 0 (any score 2–6) due to few values greater than 1. The set of values (1 vs. 0) from each animal for days 1–5 was analyzed using generalized estimating equations specifying a binomial distributional with an exchangeable correlation structure using the GENMOD procedure in SAS<sup>®</sup> (SAS Institute, Inc., Cary, NC). A main effects model of group and day was fit. The data were too sparse to fit the group × day interaction. To compare the relative likelihoods of one group on average having behavior level 1 relative to another group (averaged across days), the model parameters were exponentiated to obtain odds ratios (ORs) and 95% confidence intervals (CIs).

Morris water maze latency, speed, and path length are expressed as mean ± SEM and were analyzed using repeated-measures analyses utilizing restricted maximum likelihood estimation to obtain parameter estimates using the MIXED procedure in SAS<sup>®</sup>. Each set of measurements from the same animal was considered a correlated cluster of observations. An unstructured covariance structure was used. *Post hoc* comparisons of means were performed using the Tukey procedure with a two-sided  $\alpha$  level of significance of 0.05.

Beam walk latency data are expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) for day 0 and repeated-measures ANOVA for PIDs 1–5.

**Physiologic Data.** Data are expressed as mean ± SD. Mean arterial pressure and other physiologic measures (arterial oxygen tension [PaO<sub>2</sub>], arterial carbon dioxide tension [Paco<sub>2</sub>], blood pH, blood glucose, hematocrit, hemoglobin, and temperatures) were analyzed using repeated-measures analyses utilizing restricted maximum likelihood estimation to obtain parameter estimates using the MIXED procedure in SAS<sup>®</sup>. Each set of measurements from the same animal was considered a correlated cluster of observations. An unstructured covariance structure was used. *Post hoc* comparisons of means were performed using Tukey procedure with a two-sided  $\alpha$  level of significance of 0.05.

**Neuronal Counts.** Data are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by a Tukey procedure using a two-sided  $\alpha$  level of significance to determine differences between groups.

All statistical analyses were conducted using SAS<sup>®</sup> (SAS/STAT<sup>®</sup> 9.1 User's Guide, Cary, NC).

## Results

### Physiologic Measures

**Mean Arterial Pressure.** There were no significant differences in MAP among the groups at 0 and 90 min after TBI (0 min:  $P = 0.75$ ; 90 min:  $P = 0.47$ ). Repeated-measures ANOVA on the 5-, 45-, and 60-min time points showed no differences between groups that received hemorrhage ( $P = 0.11$ ). The amount of blood withdrawn to achieve and maintain the lowered MAP did not differ among groups (THS: 7.1 ± 0.8 ml; THH: 6.4 ± 1.7 ml; THA: 7.3 ± 1.9 ml;  $P = 0.47$ ; table 1).

**Blood Gases, pH, and Glucose.** Repeated-measures ANOVA revealed no intergroup differences for Po<sub>2</sub> ( $P = 0.42$ ), Pco<sub>2</sub> ( $P = 0.07$ ), pH ( $P = 0.55$ ), and blood glucose levels ( $P = 0.96$ ) at any of the time points (table 2).

**Hematocrit and Hemoglobin.** For hematocrit, there was a significant overall effect of treatment ( $P = 0.01$ ) and time ( $P < 0.0001$ ) as well as a significant interaction (treatment × time;  $P = 0.02$ ). Comparisons of treatment groups showed that there were no significant differences between treatment groups at each time point. However, in the groups that received hemorrhage (HEM, THS, THH, and THA), hematocrit was significantly lower at the 60- and 90-min time points compared with baseline ( $P < 0.05$ ; table 3). The same pattern was observed for hemoglobin. There was a significant overall effect of treatment ( $P = 0.01$ ) and time ( $P < 0.0001$ ) and a significant treatment × time interaction ( $P = 0.015$ ). There were no significant differences between treatment groups at each time point, although the groups that received hemorrhage showed significantly lower hemoglobin levels at 60 and 90 min after hemorrhage compared with baseline ( $P < 0.05$ ; table 3).

### Behavioral Assessments

**Beam Balance.** The data were summarized for each day as a 1 if all scores equaled 1 or as a 0 for any score of 2–6. This gave values of 1 or 0 for each of the 5 days.

**Table 2. Arterial Blood Gases, pH, and Plasma Glucose**

Group	0 min	60 min	90 min
<b>Po<sub>2</sub>, mm Hg</b>			
SHAM	213 ± 11	205 ± 133	209 ± 9
HEM	211 ± 27	207 ± 20	217 ± 13
TBI	217 ± 22	219 ± 17	208 ± 21
THS	219 ± 26	220 ± 21	231 ± 18
THH	219 ± 27	223 ± 28	213 ± 22
THA	209 ± 36	211 ± 21	217 ± 18
<b>Pco<sub>2</sub>, mm Hg</b>			
SHAM	36 ± 4.3	33.1 ± 3.4	34.1 ± 5.2
HEM	38.5 ± 4.1	36.3 ± 4.2	36 ± 4.8
TBI	37.1 ± 3.1	37.4 ± 1.3	37.9 ± 1.1
THS	37.6 ± 2.5	35.6 ± 2.5	34.5 ± 1.4
THH	36.5 ± 2.5	37.9 ± 1.8	37.1 ± 1.4
THA	38.6 ± 2.4	39.6 ± 3.4	35.8 ± 3.5
<b>pH</b>			
SHAM	7.47 ± 0.04	7.49 ± 0.03	7.48 ± 0.02
HEM	7.45 ± 0.04	7.45 ± 0.08	7.46 ± 0.03
TBI	7.47 ± 0.03	7.46 ± 0.02	7.47 ± 0.05
THS	7.48 ± 0.03	7.47 ± 0.06	7.48 ± 0.02
THH	7.48 ± 0.04	7.42 ± 0.04	7.43 ± 0.03
THA	7.44 ± 0.03	7.42 ± 0.06	7.42 ± 0.03
<b>Glucose, mg/dl</b>			
SHAM	187 ± 45	115 ± 7	114 ± 10
HEM	198 ± 73	147 ± 44	150 ± 29
TBI	167 ± 32	130 ± 20	130 ± 49
THS	201 ± 53	185 ± 69	156 ± 67
THH	232 ± 118	157 ± 47	168 ± 54
THA	207 ± 61	168 ± 83	170 ± 70

Arterial blood gases, pH, and plasma glucose in rats before and after moderate (2.0 atm) fluid percussion traumatic brain injury only (n = 5) or sham (n = 5) surgery and/or hemorrhagic hypotension and resuscitation with saline (n = 8), hypertonic saline (n = 8), or 100 mg/kg L-arginine in hypertonic saline (n = 8). See text for statistical analysis.

Glucose = plasma glucose; HEM = hemorrhage and resuscitation with normal saline; pH = arterial pH; Pco<sub>2</sub> = arterial carbon dioxide tension; Po<sub>2</sub> = arterial oxygen tension; SHAM = sham surgery only; TBI = traumatic brain injury only; THA = TBI plus hemorrhage and resuscitation with L-arginine in hypertonic saline; THH = TBI plus hemorrhage and resuscitation with hypertonic saline; THS = TBI plus hemorrhage and resuscitation with normal saline.

A main effects model showed significant effects for treatment group (*P* = 0.047) and day (*P* = 0.002). Individual group comparisons found that rats with TBI alone were 16 times more likely (OR, 16.42; 95% CI, 1.89–143) and rats with TBI plus hemorrhage and normal saline reperfusion were 18 times more likely to exhibit behavior level 0, indicating a poorer performance (THS; OR, 18.2; 95% CI, 2.39–139) compared with SHAM. Rats treated with hypertonic saline (THH) were 6 times more likely (OR, 6.17; 95% CI, 1.25–30.5) and rats that received hypertonic L-arginine (THA) were 3 times more likely (2.80; 95% CI, 0.922–8.48) to exhibit behavior level 1, indicating a favorable outcome, compared with saline-treated rats (THS; fig. 1).

**Beam Walk.** There were no significant differences among the treatment groups in the beam walk performance (data not shown).

**Morris Water Maze.** For latency to find the platform, a significant overall effect of treatment (*P* = 0.01) and day (*P* < 0.0001) as well as a treatment × day interaction

**Table 3. Hematocrit and Hemoglobin**

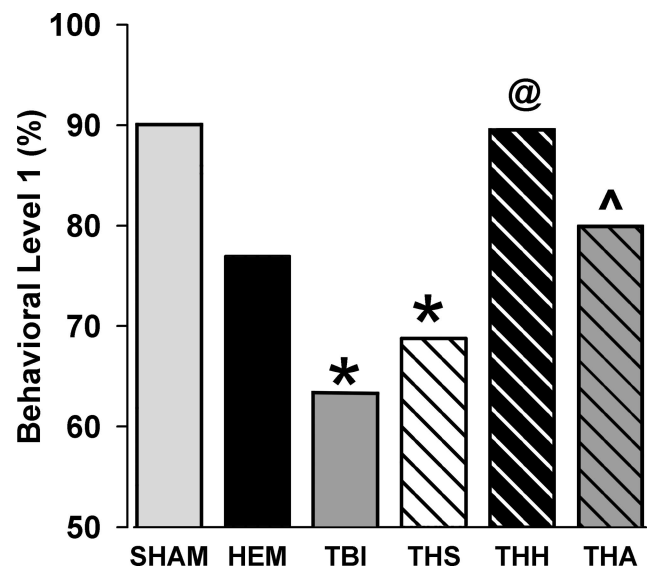
Group	0 min	60 min	90 min
<b>Hematocrit, %</b>			
SHAM	44.0 ± 1.30	43.0 ± 1.76	43.4 ± 1.33
HEM	51.0 ± 1.64	42.4 ± 1.74*	40.8 ± 1.41*
TBI	44.2 ± 1.50	43.2 ± 1.39	42.1 ± 1.71
THS	47.9 ± 2.54	41.0 ± 1.16*	39.9 ± 1.94*
THH	45.0 ± 1.64	37.0 ± 2.04*	38.5 ± 1.88*
THA	43.5 ± 1.78	36.4 ± 1.80*	34.4 ± 1.73*
<b>Hemoglobin, mg/dl</b>			
SHAM	14.7 ± 0.441	14.3 ± 0.588	14.4 ± 0.434
HEM	17.0 ± 0.547	14.1 ± 0.575*	13.6 ± 0.473*
TBI	14.7 ± 0.486	14.5 ± 0.548	14.3 ± 0.396
THS	16.0 ± 0.844	13.8 ± 0.245*	13.3 ± 0.646*
THH	15.1 ± 0.538	12.4 ± 0.678*	12.8 ± 0.627*
THA	14.5 ± 0.599	12.2 ± 0.612*	11.5 ± 0.577*

Hematocrit and hemoglobin in rats before (0 min) and after moderate (2.0 atm) fluid percussion traumatic brain injury only (n = 5) or sham (n = 5) surgery and/or hemorrhagic hypotension and resuscitation with saline (n = 8), hypertonic saline (n = 8), or 100 mg/kg L-arginine in hypertonic saline (n = 8).

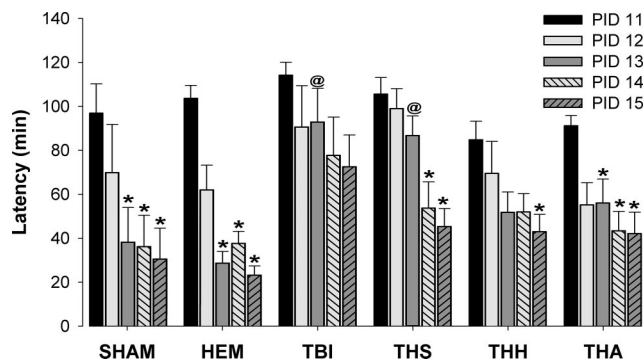
\* *P* < 0.05 compared with 0 min within treatment group.

HEM = hemorrhage and resuscitation with normal saline; SHAM = sham surgery only; TBI = traumatic brain injury only; THA = TBI plus hemorrhage and resuscitation with L-arginine in hypertonic saline; THH = TBI plus hemorrhage and resuscitation with hypertonic saline; THS = TBI plus hemorrhage and resuscitation with normal saline.

(*P* = 0.005) was observed. Comparison of individual treatment groups revealed that SHAM, HEM, and THA were the only treatment groups that showed a significantly improved performance on PIDs 13–15 compared



**Fig. 1.** Beam balance performance for all groups is presented as the percent categorized as a behavior level of 1. Each day was summarized as behavior level 1 (= 1) versus 2, 3, 4 (= 0) due to few values greater than 1 (see Materials and Methods for details) in the following treatment groups: SHAM = sham surgery; HEM = sham surgery + hemorrhage; TBI = fluid percussion injury; THS = TBI + hemorrhage + normal saline resuscitation; THH = TBI + hemorrhage + hypertonic saline resuscitation; THA = TBI + hemorrhage + L-arginine in hypertonic saline resuscitation. THH and THA groups were more likely to perform well than TBI and THS. Scores for postinjury days 1–5 are combined (\**P* < 0.05 vs. SHAM; @*P* < 0.05 vs. TBI; ^*P* = 0.06).

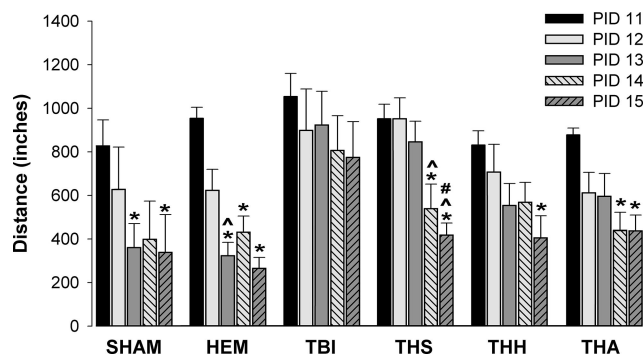


**Fig. 2.** Latency in minutes to find the hidden platform in the Morris water maze test presented as mean  $\pm$  SEM (see Materials and Methods for details) in the following treatment groups: SHAM = sham surgery; HEM = sham surgery + hemorrhage; TBI = fluid percussion injury; THS = TBI + hemorrhage + normal saline resuscitation; THH = TBI + hemorrhage + hypertonic saline resuscitation; THA = TBI + hemorrhage + L-arginine in hypertonic saline resuscitation. THH and THA groups performed better earlier after injury compared with TBI and THS. Rats were tested on postinjury days (PIDs) 11–15 (\* $P < 0.05$  vs. PID 11 within group; @ $P < 0.05$  vs. same day HEM).

with PID 11 within group ( $P < 0.05$ ; fig. 2). The TBI and THS treatment groups performed significantly worse than the HEM group on PID 13 ( $P < 0.05$ ). However, the THS group did show improved performance compared with PID 11 on PIDs 14–15, and the THH group showed an improved performance compared with PID 11 on PID 15 (within group).

A similar pattern of behavior was observed in measures of path length. A significant effect of treatment ( $P = 0.015$ ) and day ( $P < 0.0001$ ) and a treatment  $\times$  day interaction ( $P = 0.01$ ) were observed. A significant difference compared with PID 11 within groups was observed by PID 13 in the SHAM and HEM groups. The TBI group showed no decrease in path length over the course of the experiment (fig. 3). The THS group was slower to take a direct path to the platform compared with the other TBI plus hemorrhage groups (THH and THA) as suggested by significant differences between PIDs 12 and 13 compared with PIDs 14 and 15 ( $P < 0.05$ ), which indicate that on PIDs 12 and 13 the path length had not decreased compared with PID 11. However, by PID 14, the THS group did show a similar reduction in path length as the other TBI plus hemorrhage groups. A significant response compared with PID 11 was observed in the THA group by PID 14 and in the THH group by PID 15. In summary, resuscitation with hypertonic L-arginine after TBI plus hemorrhage seemed to improve performance in the Morris water maze as measured by both latency and distance traveled to find the hidden platform.

There were no differences in swimming speed between treatment groups ( $P = 0.73$ ), indicating that motor impairment was not a factor in the Morris water maze outcomes (table 4).



**Fig. 3.** Path length in inches to find the hidden platform in the Morris water maze test presented as mean  $\pm$  SEM (see Materials and Methods for details) in the following treatment groups: SHAM = sham surgery; HEM = sham surgery + hemorrhage; TBI = fluid percussion injury; THS = TBI + hemorrhage + normal saline resuscitation; THH = TBI + hemorrhage + hypertonic saline resuscitation; THA = TBI + hemorrhage + L-arginine in hypertonic saline resuscitation. THH and THA groups performed better earlier after injury compared with TBI and THS. Rats were tested on postinjury days (PIDs) 11–15 (\* $P < 0.05$  vs. PID 11 within group; ^ $P < 0.05$  vs. PID 12 within group; # $P < 0.05$  vs. PID 13 within group).

### Neuronal Counting

No significant overall effects were observed in the hippocampal areas ipsilateral to the site of injury (CA1,  $P = 0.07$ ; CA2–3,  $P = 0.64$ ; hilus,  $P = 0.17$ ; total,  $P = 0.08$ ; fig. 4A). However, in the contralateral hippocampus, an overall effect of treatment was detected in areas CA1 ( $P = 0.01$ ) and CA2–3 ( $P = 0.02$ ) as well as for total counts ( $P = 0.02$ ). There was no significant effect in the contralateral hilus ( $P = 0.36$ ). Individual group comparisons in the contralateral hippocampus revealed significantly larger numbers of live neurons in both TBI plus hemorrhage groups that were treated with hypertonic saline or hypertonic L-arginine (THH and THA) compared with the THS group in areas CA1 and CA2–3 as well as total counts ( $P < 0.05$ ; fig. 4B).

### Discussion

These experiments are the first to address the hypothesis that hypertonic saline resuscitation fluids with or without added L-arginine enhance behavioral outcomes and neuronal survival in a rat model of combined TBI and hemorrhage. In this study, motor and cognitive deficits caused by TBI were slightly reduced by post-TBI hemorrhage plus normal saline resuscitation. However, administration of hypertonic (7.5%) saline and hypertonic L-arginine improved behavioral outcome in comparison with resuscitation with an equal volume of 0.9% saline. Furthermore, rats resuscitated with hypertonic L-arginine had a slightly better cognitive outcome compared with those that received hypertonic saline.

Histologic outcome after moderate TBI also was not aggravated by post-TBI hemorrhage in this study. However, while no significant effect of treatment was observed in the ipsilateral hippocampus, in the contralat-

**Table 4. Morris Water Maze Swim Speeds**

Group	PID 11	PID 12	PID 13	PID 14	PID 15
SHAM	8.5 ± 0.58	9.3 ± 0.90	9.2 ± 0.72	9.9 ± 0.77	9.5 ± 1.0
HEM	9.4 ± 0.41	10.3 ± 0.36	10.9 ± 0.29	11.2 ± 0.44	11.0 ± 0.43
TBI	9.2 ± 0.62	10.0 ± 0.57	9.7 ± 0.89	10.8 ± 1.4	10.6 ± 0.91
THS	9.0 ± 0.46	9.5 ± 0.52	9.5 ± 0.41	9.6 ± 0.65	10.6 ± 1.3
THH	9.8 ± 0.31	9.6 ± 0.32	10.6 ± 0.40	10.7 ± 0.32	10.9 ± 0.43
THA	9.5 ± 0.26	10.6 ± 0.38	10.9 ± 0.42	10.3 ± 0.24	10.6 ± 0.52

Morris water maze swim speeds (inches/seconds) in rats before and after moderate (2.0 atm) fluid percussion traumatic brain injury only (n = 5) or sham (n = 5) surgery and/or hemorrhagic hypotension and resuscitation with saline (n = 8), hypertonic saline (n = 8), or 100 mg/kg L-arginine in hypertonic saline (n = 8). No differences between treatment groups (*P* = 0.73).

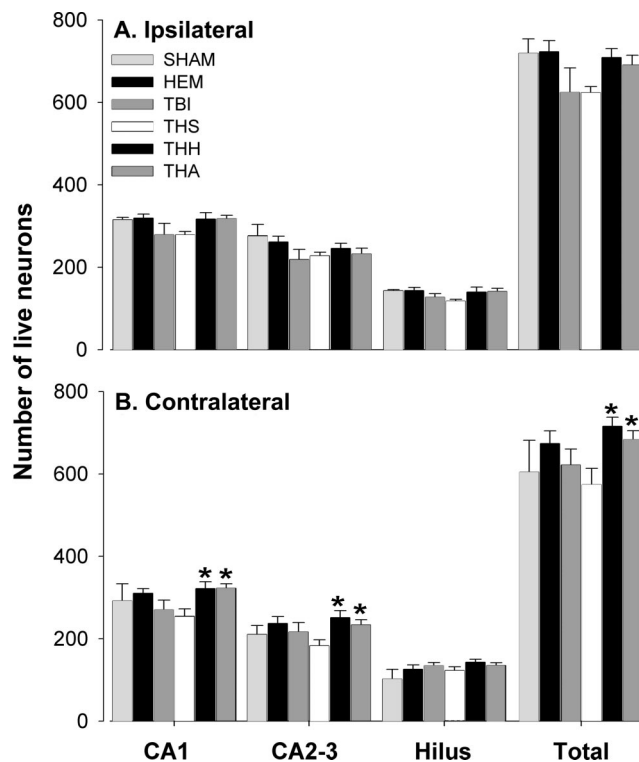
HEM = hemorrhage and resuscitation with normal saline; PID = postinjury day; SHAM = sham surgery only; TBI = traumatic brain injury only; THA = TBI plus hemorrhage and resuscitation with L-arginine in hypertonic saline; THH = TBI plus hemorrhage and resuscitation with hypertonic saline; THS = TBI plus hemorrhage and resuscitation with normal saline.

eral hippocampus, in comparison with resuscitation with 0.9% saline, both hypertonic saline and hypertonic L-arginine reduced neuronal loss. This reduced neuronal loss in the contralateral hippocampus may be due to the regional nature of trauma-induced hypoperfusion. Although CBF reductions due to parasagittal fluid percussion TBI are bilateral, they tend to be more pronounced and recover more slowly on the ipsilateral side.<sup>28</sup> If

trauma-induced reductions in CBF were less profound on the contralateral side, hypertonic resuscitation may have restored CBF to levels sufficient to spare more hippocampal neurons in that hemisphere. However, because CBF was not measured in this study, this hypothesis remains speculative.

Although the addition of L-arginine to hypertonic saline did not enhance neuronal survival and only slightly enhanced cognitive performance over that of hypertonic saline alone, there was no indication of any adverse effect. In fact, rats that received TBI plus hemorrhage and were treated with hypertonic L-arginine were most similar in outcome to rats that received sham surgery or hemorrhage only (no TBI), suggesting a therapeutic potential for this compound.

Systemic hypotension after TBI is a common occurrence that contributes to up to three times the mortality rate compared with brain-injured patients who do not experience hypotension.<sup>2,3</sup> Although the optimal resuscitation protocol for brain-injured patients with hypotension has yet to be determined, current literature strongly supports a role for the use of hypertonic saline. While the American College of Surgeons recommends the rapid infusion of 2 liters Ringer's lactate or 0.9% saline, several serious complications are possible, such as displacement of hemostatic clots, hemodilution, and increased secondary blood loss.<sup>6</sup> In fact, the Advanced Trauma Life Support course advises caution in the use of fluid in treating TBI patients for fear that fluid delivery could increase cerebral edema and ICP. Mannitol is routinely used in the clinical setting for the purpose of reducing ICP; however, mannitol can lead to volume deficits and increased risk of systemic hypotension, thus increasing the risk of secondary cerebral ischemic injury. In contrast, hypertonic saline has been shown to reduce ICP without reducing systemic blood pressure and thus enhance survival in trauma patients compared with those who received isotonic saline solutions.<sup>8</sup> In fact, patients with torso trauma who received 7.5% saline with dextran were twice as likely to survive as those who received 0.9% saline.<sup>29</sup>



**Fig. 4.** Counts of live neurons in the hippocampus ipsilateral (A) and contralateral (B) to the site of the injury presented as mean ± SEM. Tissue was collected on postinjury day 15 immediately after completion of behavioral testing (see Materials and Methods for details) in the following treatment groups: SHAM = sham surgery; HEM = sham surgery + hemorrhage; TBI = fluid percussion injury; THS = TBI + hemorrhage + normal saline resuscitation; THH = TBI + hemorrhage + hypertonic saline resuscitation; THA = TBI + hemorrhage + L-arginine in hypertonic saline resuscitation. In the contralateral hemisphere, THH and THA groups had increased neuronal survival compared with the THS group (\**P* < 0.05 vs. THS).

Improved survival after treatment with hypertonic saline is dependent on either previous hemostasis or delaying fluid administration until the time of surgery when hemostasis can be ensured.<sup>30</sup> Because our model consists of hypotension that is initiated by controlled removal of blood, and resuscitation occurs only after the blood has been removed and there is no further bleeding, our model is similar to the situation where hemostasis has been established. In this case, treatment with 7.5% saline alone or with L-arginine improves neuronal survival and cognitive performance after injury.

We and others have previously demonstrated many advantageous effects of resuscitation with L-arginine. For example, L-arginine prevents or reverses posttraumatic cerebral hypoperfusion<sup>15</sup> and increases CBF without altering cerebral perfusion pressure. L-Arginine has also been shown to reduce contusion volume<sup>16</sup> and increase blood flow and nitric oxide concentrations in a severe cortical impact injury model in rats.<sup>17</sup> Furthermore, L-arginine produced favorable effects on the heart rate and tissue oxygenation in hemorrhaged rabbits<sup>31</sup> and increased survival in hemorrhaged rats.<sup>12</sup> In the same TBI/hemorrhage model that was used in the current study, both hypertonic saline and hypertonic L-arginine improved MAP and CBF equally, but long-term outcome was not examined.<sup>18</sup> Therefore, the current study was designed to determine whether acute improvements in MAP, ICP, and CBF in response to hypertonic resuscitation were associated with increased neuronal survival and improved behavioral outcomes 15 days after TBI and hemorrhage.

These studies also were intended to explore the possibility that treatment with hypertonic saline might be neurotoxic rather than neuroprotective. After TBI, inducible nitric oxide synthase production increases, potentially producing excess nitric oxide, which can lead to production of peroxynitrite, a powerful oxidant.<sup>32</sup> This potential neurotoxic role for nitric oxide is supported by evidence that treatment with inducible nitric oxide synthase inhibitors enhances survival in a TBI-hemorrhage model.<sup>33</sup> Therefore, it was important to determine whether treatment with hypertonic L-arginine was associated with improved or worsened neuronal survival 15 days after TBI and hemorrhage.

In this study, we tested only one dose and treatment regimen of L-arginine. Because we did not measure production of nitric oxide or peroxynitrite, we cannot say with certainty that the concentration of L-arginine that was achieved actually increased either compound. It is possible that the reason we did not observe large differences between treatments with hypertonic saline alone or hypertonic L-arginine could be because the concentration of L-arginine was too small to effectively enhance production of nitric oxide. Furthermore, because we did not directly measure CBF, we cannot demonstrate that CBF was any greater for the group treated with L-arginine

versus hypertonic saline alone. Although in a previous study hypertonic L-arginine significantly reduced ICP compared with hypertonic saline, there were no differences in MAP or CBF between the two hypertonic solutions for 90 min after resuscitation.<sup>18</sup>

Our current results, that hypertonic L-arginine and hypertonic saline equally improve neuronal survival and cognitive outcomes at 15 days after injury, are consistent with our previous findings that hypertonic saline and hypertonic L-arginine had similar effects on CBF and ICP. It is important to note, however, that there was no evidence of increased cellular, neuronal, or vascular damage with the addition of L-arginine. Therefore, further studies are still warranted to investigate the conflicting results regarding inclusion of L-arginine in resuscitation protocols after trauma.

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