Effect of Glutamate and Blood Glutamate Scavengers Oxaloacetate and Pyruvate on Neurological Outcome and Pathohistology of the Hippocampus after Traumatic Brain Injury in Rats

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ABSTRACT

Background: Decreasing blood glutamate concentrations after traumatic brain injury accelerates brain-to-blood glutamate efflux, leading to improved neurologic outcomes. The authors hypothesize that treatment with blood glutamate scavengers should reduce neuronal cell loss, whereas administration of glutamate should worsen outcomes. The authors performed histologic studies of neuronal survival in the rat hippocampus after traumatic brain injury and treatment with blood glutamate scavengers.

Methods: Traumatic brain injury was induced on anesthetized male Sprague-Dawley rats by a standardized weight drop. Intra-venous treatment groups included saline (control), oxaloacetate, pyruvate, and glutamate. Neurologic outcome was assessed using a Neurological Severity Score at 1 h, and 1, 2, 7, 14, 21, 28 days. Blood glutamate was determined at baseline and 90 min.

Results: Oxaloacetate and pyruvate treatment groups demonstrated increased neuronal survival (oxaloacetate 2,200 ± 37, pyruvate 2,108 ± 137 vs. control 1,978 ± 46, P < 0.001, mean ± SD). Glutamate treatment revealed decreased neuronal survival (1,715 ± 48, P < 0.001). Treatment groups demonstrated favorable neurologic outcomes at 24 and 48 h (Neurological Severity Score at 24 and 48 h: 5.5 (1–8.25), 5 (1.75–7.25), P = 0.02 and 3(1–6.5), 4 (1.75–4.5), P = 0.027, median ± corresponding interquartile range). Blood glutamate concentrations were decreased in the oxaloacetate and pyruvate treatment groups. Administration of oxaloacetate and pyruvate was not shown to have any adverse effects.

Conclusions: The authors demonstrate that the blood glutamate scavengers oxaloacetate and pyruvate provide neuroprotection after traumatic brain injury, expressed both by reduced neuronal loss in the hippocampus and improved neurologic outcomes. The findings of this study may bring about new therapeutic possibilities in a variety of clinical settings.

What We Already Know about This Topic

• Decreasing blood glutamate concentrations after traumatic brain injury (TBI) can accelerate brain-to-blood glutamate efflux, leading to improved neurologic outcomes

What This Article Tells Us That Is New

• In a rat model, the blood glutamate scavengers oxaloacetate and pyruvate provide neuroprotection after TBI

Four weeks after traumatic brain injury, a histologic analysis of surviving neurons was performed.

ABNORMALLY increased glutamate concentrations in the brain’s extracellular fluid (ECF) and cerebrospinal fluid are observed in the context of several acute neurodegenerative conditions, including traumatic brain injury (TBI),1–5 and have been demonstrated to correlate with poor neurologic outcomes in human and animal studies.6–13 The brain has several inherent mechanisms by which it removes...
excess glutamate. In one such mechanism, a large family of Na+-dependent glutamate transporters on nerve terminals and astrocytes facilitate the uptake of glutamate into brain cells, ensuring that excess concentrations of glutamate in brain ECFs are reduced to concentrations at which it is no longer active or toxic.14,15 A second glutamate removal pathway is facilitated by glutamate transporters present on the antiluminal side of the brain capillary endothelial cells,16,17 thus eliminating glutamate from the brain into the blood.14,16,18,19 Gottlieb et al.20 demonstrated the existence of this brain-to-blood efflux by injecting radiolabeled glutamate into rat lateral cerebral ventricles and subsequently showing its rapid appearance in the blood and disappearance from the cerebrospinal fluid. The rate of this brain-to-blood efflux of glutamate was increased by creating a larger concentration gradient between the brain ECF or cerebrospinal fluid and blood.20 A decrease in blood glutamate concentrations, increasing the driving force for the brain-to-blood glutamate efflux, was achieved by exploiting the glutamate-scavenging properties of the blood resident enzymes glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase, which in the presence of their glutamate substrates oxaloacetate and pyruvate, convert glutamate into its inactive form 2-ketoglutarate.20 Dual-probe brain microdialysis studies in rats demonstrated an oxaloacetate-mediated reduction of blood glutamate, correlating to striatum ECF glutamate concentrations increasing when plasma glutamate concentrations were increased.17 The reduction of blood glutamate concentrations correlated with significant neurologic improvement after TBI.21 Pyruvate has been shown to possess similar glutamate scavenging properties.22

Previous studies in our laboratory established the motor and behavioral effects of blood glutamate manipulation, either by scavenging or injecting glutamate in a rat model of TBI.23,24 This was assessed using the Neurological Severity Score (NSS), which is subjective to the examiner. To date, we have not quantitatively assessed the effect on neuronal damage in the brain.

Several processes are known to evolve after TBI and are evident on histologic examination, including brain edema, neuronal necrosis, and apoptosis.25 The hippocampus in particular is known to be sensitive to the effects of ischemia and TBI. Thus, ischemic and traumatic insults result in neuronal loss in different hippocampal regions. We hypothesize that neuronal cell loss after TBI should be reduced when TBI is treated with blood glutamate scavengers. Conversely, when glutamate is administered intravenously, the deleterious effects on neuronal loss should be aggravated. In the current study, we performed a histologic examination of rat brain slices, assessing histologic outcome after TBI in control and treatment groups. Accordingly, we calculated the amount of surviving neurons in different regions of the hippocampus (CA1 through CA4) and dentate gyrus.26–28 In addition, we studied the impact of artificially increasing blood glutamate concentrations by injecting glutamate into the blood after TBI.

Materials and Methods

The experiments were conducted according to the recommendations of the Declarations of Helsinki and Tokyo and in adherence with the Guidelines for the Use of Experimental Animals of the European Community. The experiments were approved by the Animal Care Committee of Ben-Gurion University of the Negev (Joint Animal Care Committee for Ben-Gurion University and Soroka Medical Center, Beer-Sheva, Israel).

Rat Model

Spontaneously breathing, male Sprague-Dawley rats weighing 200–300 g were anesthetized with a mixture of isoflurane (initial inspired concentration 2%) in 100% oxygen (1 l/min). A heating pad was used to maintain the rectal temperature at 37°C, and anesthesia was considered sufficient for surgery when the tail reflex was abolished. All rats were divided into two cohorts: the sham cohort received a scalp incision and insertion of arterial and venous lines with no TBI, and the TBI cohort underwent the same and was also exposed to TBI. From the initial TBI cohort, 11 rats were excluded because the head injury was not classified as “moderate” (NSS 10–21). After the neurologic assessment and application of the exclusion criteria, 29 rats were studied. After TBI, the rats were assigned randomly to one of four treatment groups: control group (isotonic saline treatment) and the oxaloacetate, pyruvate, and glutamate groups. All groups consisted of six rats, with the exception of the control group, which included five rats. Drugs were administered intravenously at a dose of 1 ml 1 M solution per 100 g of weight, starting 60 min after TBI at a continuous infusion rate of 1 ml · 100 g⁻¹ · min⁻¹ during 30 min.

Vascular Access

Catheterization of the tail vein was carried out with a 24-g BD Neoflon catheter (Becton, Dickinson and Company, Franklin Lakes, NJ) for administration of fluids and drug infusions. Catheterization of the tail artery was performed to allow blood sampling and measurement of blood pressure and heart rate. Mean arterial blood pressure was determined by electronic integration.

Blood Sampling

Arterial blood samples were drawn at t = 0 (baseline) and at 60 and 90 min after head injury. Samples were analyzed for arterial blood gas tension, hemoglobin, glutamate, and glucose concentrations.

TBI

With the animal under general anesthesia, the scalp was infiltrated with 0.5% bupivacaine. The scalp was incised and reflected laterally, and a cranial impact of 0.5 J was delivered.
by a silicone-coated rod protruding from the center of a free-falling plate, as described previously.21–23 The impact point was 1–2 mm lateral to the midline of the skull’s convexity. After TBI, the incision was sutured, and the rat was laid on the left side for recovery from anesthesia, which took place within 60 min after TBI.

**Neurological Severity Score.** The NSS was determined by a blinded observer.23 Points were assigned for alterations of motor function and behavior, such that the maximal score of 25 represents greatest neurologic dysfunction, whereas a score of 0 indicates an intact neurologic condition. Specifically, the following were assessed: ability to exit from a circle (3-point scale), gait on a wide surface (3-point scale), gait on a narrow surface (4-point scale), effort to remain on a narrow surface (2-point scale), reflexes (5-point scale), seeking behavior (2-point scale), beam walking (3-point scale), and beam balance (3-point scale). Assessment of the NSS was done in two phases. The acute phase (within the first 48 h) was expected to yield the most significant changes. The second phase, representing long-term outcomes, was evaluated at 1, 2, 3, and 4 weeks.

**Experimental Design**

Traumatic brain injury was delivered at \( t = 0 \) min, immediately after collection of the first baseline blood sample. The first assessment of the NSS was performed at \( t = 60 \) min. Only animals with NSS between 10 and 21, corresponding to moderate-severe head injury, were included in the study. Previous studies based on this model have reported that when NSS is more than 21, neurologic injury is so severe that no treatment significantly improves the neurologic outcome. When NSS is 0–10, the neurologic injury is so mild that the NSS significantly improves over time with or without treatment.23

**Drug Treatment**

Oxaloacetate, pyruvate, and glutamate were purchased from Sigma–Aldrich (St. Louis, MO). All of the treatment solutions were prepared to 1 M concentration by dilution in sterile water. The first group received intravenous infusion over 30 min of 1 ml/100 g isotonic saline. The second, third, and fourth groups received intravenous infusion over 30 min of 1 ml/100 g 1 M oxaloacetate, pyruvate, and glutamate, respectively. Dosing and timing of treatment were guided by the findings of previous studies.20,21,24

Arterial and venous catheters were removed after the last blood samples were collected 120 min after TBI; the removals did not require additional anesthesia. The animals were returned to their cages and allowed free access to food and water. Assessment of the NSS was repeated at 24 and 48 h after TBI and at 7, 14, 21, and 28 days after TBI. The difference between NSS 1 h after TBI and the later assessments served as a record of neurologic improvement.23

**Glutamate Determination**

Blood samples were drawn at \( t = 0 \) min, just before TBI (the baseline glutamate concentration immediately after induction of anesthesia and arterial line placement), and at \( t = 90 \) min (completion of intravenous drug or saline infusion). These time points were chosen based on data from our previous experiments, which demonstrated that changes in blood glutamate concentrations correlate best to the extent of neurologic improvement within 90 min after conclusion of treatment after TBI.21,22,24 Whole blood (200 \( \mu l \) aliquot) was deproteinized by adding an equal volume of ice-cold 1 M perchloric acid and centrifuging at 10,000 g for 10 min at 4°C. The pellet was discarded and supernatant collected, adjusted to \( pH \) 7.2 with 2 M \( K_2CO_3 \), and, if needed, stored at −80°C for later analysis. The glutamate concentration was measured using the fluorometric method of Graham and Aprison.29 A 20-\( \mu l \) aliquot from the perchloric acid supernatant was added to 480 \( \mu l \) 0.3 M glycine, 0.25 M hydrazine hydrate buffer adjusted to \( pH \) 8.6 with 1 M \( H_2SO_4 \) and containing 15 U glutamate dehydrogenase in 0.2 mM nicotinamide adenine dinucleotide. After the sample was incubated for 30–45 min at room temperature, its fluorescence was measured at 460 nm with excitation at 350 nm. A glutamate standard curve was established, with concentrations ranging from 0 to 6 \( \mu M \). All determinations were done at least in duplicates.

**Statistical Analysis**

Statistical analysis was performed with PASW (SPSS) version 18 (SPSS Inc, Chicago, IL). We divided our analysis into two time periods: the acute time period was defined as NSS and cell viability at 1, 24, and 48 h, and the overall period included NSS and cell viability at 1, 24, and 48 h and 1, 2, 3, and 4 weeks. Bonferroni post hoc testing was performed only when several comparisons were made on the same parameter.

**Acute Period**

To assess improvement of the NSS from 1 h to 24 and 48 h, we used nonparametric comparisons, the Wilcoxon signed-rank test. The level of significance with Bonferroni correction was \( P < 0.025 \), which was established by dividing 0.05 to the number of comparisons made. Because we conducted two comparisons (1 vs. 24 h, 24 vs. 48 h), we divided 0.05 by 2. The NSS descriptives are presented as median values and the corresponding range (interquartile range).

**Overall Period**

Analysis of the NSS at 1, 24, and 48 h and 1, 2, 3, and 4 weeks was made using a Generalized Estimation Equation (GEE) model, which allows for correlation without defining the exact dependency between the variables entered in the model to estimate the average response of the population. We included the NSS at 1, 24, and 48 h and 1, 2, 3, and 4 weeks; because the measurements are taken from the same animal at different time points, they are not independent of...
followed by buffered formaldehyde (buffered CH2O4 % w t brains were fixated by perfusion through the heart with saline 5%, for 5 min, and 3 min after tail reflex was abolished, the Four weeks after TBI, rats were anesthetized with isoflurane,

Pathohistologic Examination
Four weeks after TBI, rats were anesthetized with isoflurane, 5%, for 5 min, and 3 min after tail reflex was abolished, the brains were fixated by perfusion through the heart with saline followed by buffered formaldehyde (buffered CH2O 4%wt and methanol 1%wt, pH 6.8–7.2.) and placed into formalin solution, 10%, for 48 h. The coronal sections of brain tissue were embedded in paraffin, processed, and cut with a microtome in 5-μm sections. Slides were deposited on glass slides and stained with hematoxylin and eosin and examined under magnification at ×40 with a ×10 objective lens using an Olympus BX40 (Olympus America Inc., Center Valley, PA) microscope by an experienced neurohistopathologist, who was blinded to control, treatment, and sham groups. Sections of hippocampus were observed with a light microscope, and regions containing hippocampal formation, including CA1–CA4 and the dentate gyrus, were chosen. Neurons were counted “manually” in the designated regions using Olympus Eyepiece Micrometer 24-mm square 10 mm/10 units (Olympus America Inc.), resulting in a square surface: EpM = 100 μM² each. The number of neurons was calculated in six squares for each region. The total surface was equal for each region and totaled 600 EpM or 600 μM². Figure 1 demonstrates a typical cell count using this method.

Results
Blood Glutamate Concentrations
Blood glutamate concentrations of the different TBI groups are shown in table 1. At baseline, there were no significant differences observed between treatment groups. Blood glutamate concentrations were decreased significantly at 90 min after TBI (time point corresponding with completion of the treatment) in the oxaloacetate and pyruvate treatment groups, compared with baseline concentrations of the same groups or compared with the saline or glutamate treatment groups at the same time point. As expected, blood glutamate concentrations were significantly increased in the glutamate treatment group after treatment compared with baseline concentrations or compared with other TBI groups at the same time point (at 90 min or after completion of the treatment).

Neurologic Outcome
The NSS was assessed in two phases. The acute phase, represented by neurologic outcomes within the first 48 h, and the chronic phase, represented by neurologic outcomes in the course of 4 weeks after injury.

The improvement in NSS during the first 48 h in all groups is shown in table 2. There were no differences in the NSS between treatment and control groups 1 h after TBI (because treatment was started only after the first NSS assessment). At 24 h after TBI, the neurologic status improved significantly (lower NSS) in the treatment groups, whereas the control group did not have significant neurologic improvement (P = 0.17). At 48 h after TBI, control and glutamate groups similarly showed no significant improvement, whereas the oxaloacetate and pyruvate treatment groups demonstrated a trend toward improvement. Consistent with our previous findings,21,22,24 the treatment with oxaloacetate and pyruvate improved neurologic outcomes at 24 and 48 h after TBI.

Figure 2 demonstrates the long-term neurologic outcome in the period between the first and fourth weeks after TBI. Consistent with our previous findings,21,22 neurologic recovery over time was observed in all groups, regardless of the treatment modality, with the greatest improvement within the first 48 h after TBI. However, as seen in table 3 using the GEE modeling method, the overall neurologic outcome was significantly better for the pyruvate treatment group than control group (−2.89 ± 1.03, CI −5.63 to −0.15). Compared with the glutamate treatment group,
the overall neurologic outcomes of the oxaloacetate and pyruvate groups are significantly better (−3.26 ± 1.08, CI −6.12 to −0.40, P = 0.016 and −3.64 ± 1.09, P = 0.005, CI −6.52 to −0.76).

**Brain Histologic Examination**

Surviving neurons in the hippocampus were calculated separately in different regions. A consistent pattern was recognized in all regions examined. The results are summarized according to hippocampal region in figure 3. Representative findings are demonstrated in figures 4 and 5. Table 4 presents a primary outcomes comparison of the viability of brain cells across all hippocampal regions.

**Control Group.** The number of surviving neurons was significantly decreased compared with the sham (naïve rats) group (P < 0.001).

**Glutamate Treatment Group.** A consistent neurodegenerative pattern was found, and the number of surviving neurons was significantly reduced compared with the sham and control groups (P < 0.001). The surviving neuron count was significantly decreased compared with the treatment groups of oxaloacetate and pyruvate (P < 0.001).

**Oxaloacetate and Pyruvate Treatment Groups.** The amount of surviving neurons was significantly decreased in all TBI groups, including the treatment groups, compared with sham (naïve) rats (P = 0.001). Nevertheless, the surviving neuronal count in treatment groups remained increased compared with control or glutamate groups (P < 0.001). Within the treatment groups, there was no significant difference in surviving neurons between the pyruvate and oxaloacetate groups (P = 1).

**Physiologic Monitoring**

With regard to the physiologic parameters, mean arterial blood pressure, heart rate, arterial blood gas values (including pH, HCO3, PaCO2), and hemoglobin were not found to have significant statistical differences at corresponding time points in TBI groups.

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**Table 1. Blood Glutamate Levels**

<table>
<thead>
<tr>
<th></th>
<th>Baseline (µMol/l)</th>
<th>90 min (after Treatment) (µMol/l)</th>
<th>P Value (Compared with Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>175 (136–203)</td>
<td>157 (136–174)</td>
<td>0.08</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>155 (135–180)</td>
<td>111 (93–144)</td>
<td>0.028</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>125 (122–156)</td>
<td>94 (84–114)</td>
<td>0.027</td>
</tr>
<tr>
<td>Glutamate</td>
<td>153 (122–180)</td>
<td>1,677 (1,461–2,197)</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Blood glutamate level (µMol/l) at 0 and 90 min after traumatic brain injury (TBI; also representing completion of the treatment). Analysis was performed using nonparametric tests (Wilcoxon signed-rank). Data are presented as median (IQ). There were no statistically significant differences between groups at baseline. There was a significant decrease of blood glutamate at 90 min in the oxaloacetate and pyruvate treatment groups compared with baseline values. There was a significant increase of blood glutamate at 90 min in the glutamate treatment group.

IQ = interquartile.

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**Table 2. Neurologic Severity Score, Acute Phase**

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>IQ</th>
<th>1 vs. 24 h, P Value</th>
<th>1 vs. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N = 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS 1 h</td>
<td>15</td>
<td>11.5–17</td>
<td>0.17</td>
<td>0.138</td>
</tr>
<tr>
<td>NSS 24 h</td>
<td>9</td>
<td>6–17.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NSS 48 h</td>
<td>9</td>
<td>5–14.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glutamate (N = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS 1 h</td>
<td>16</td>
<td>14–17.75</td>
<td>0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>NSS 24 h</td>
<td>11</td>
<td>8.5–15.75</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NSS 48 h</td>
<td>9.5</td>
<td>6–14.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oxaloacetate (N = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS 1 h</td>
<td>15.5</td>
<td>13.25–16.75</td>
<td>0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>NSS 24 h</td>
<td>5.5</td>
<td>1–8.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NSS 48 h</td>
<td>3</td>
<td>1–6.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pyruvate (N = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS 1 h</td>
<td>15</td>
<td>11.5–16.5</td>
<td>0.02</td>
<td>0.027</td>
</tr>
<tr>
<td>NSS 24 h</td>
<td>5</td>
<td>1.75–7.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NSS 48 h</td>
<td>4</td>
<td>1.75–4.5</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Improvement of the Neurological Severity Score (NSS) in the initial 48 h after injury. The NSS was assessed at 1, 24, and 48 h after traumatic brain injury using a scale of 0–25 points, in which 0 represents no neurologic impairment and 25 represents maximal neurologic impairment. N is the number of rats/group. The NSS scores are presented as median values and the corresponding range (IQ). The data were analyzed by the Wilcoxon signed-rank test. The level of significance for these comparisons is 0.025, due to a Bonferroni correction for multiple (i.e., 2) comparisons (1 vs. 24 h, 1 vs. 48 h).

IQ = interquartile.
Blood Glucose

Blood glucose concentrations at $t = 0$ and 90 min were not found to be statistically significantly different in the TBI groups at corresponding time points or compared with baseline values in the same TBI groups.

Discussion

This study demonstrated for the first time the effect of the blood glutamate scavengers oxaloacetate and pyruvate on cell survival in hippocampus of the rat after TBI. Thirty days after injury to the rats, we observed a significantly larger number of surviving neurons in different hippocampal regions in rats treated with a 30-min IV infusion of oxaloacetate and pyruvate carried out 60 min after the injury. This correlates closely with the transient decrease in blood glutamate concentrations at 90 min after TBI and the improved neurologic outcomes of these rats in the first days after injury. Conversely, a transient artificial increase of plasma glutamate by intravenous infusion of glutamate led to significantly decreased neuronal survival in different regions of hippocampus. Diminished neuronal loss in the hippocampus (which is known to involve behavior, learning, and memory) in treated rats and its association with improved neurologic outcomes provide an insight into the underlying neuroprotective mechanism of blood glutamate scavengers.23

We previously established the beneficial neurologic effects of blood glutamate scavengers by demonstrating a correlation between the treatment with blood glutamate scavengers and subjective findings (behavior and motor scores that are part of the NSS). Although the effect of blood glutamate scavengers on neurologic outcomes is most prominent in the first days after injury (the acute phase), the effect on neuronal survival in the hippocampus manifests itself later. The transient activity of oxaloacetate was shown to act as a neuroprotective drug, leading to improved neurologic recovery within the first 48 h after injury and providing protection from hippocampal neuronal loss at 30 days after TBI in rats. Although neuronal loss is diminished in treated rats compared with controls, the hippocampal neuronal loss remains increased compared with naive rats, thus offering partial neuroprotection.

Surviving neuronal count in the hippocampus is a method widely used for quantitative histopathologic assessment of injury to the brain after TBI.26–28 TBI-induced brain hypoperfusion may produce cerebral ischemia involving the hippocampus, in which specific deficits have been mapped to certain

Table 3. Generalized Linear Model of Neurologic Outcomes

<table>
<thead>
<tr>
<th>(I) Group1</th>
<th>(J) Group1</th>
<th>Mean Difference (I-J)</th>
<th>SE</th>
<th>Bonferroni Significance</th>
<th>95% CI for Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>Oxaloacetate</td>
<td>−.38</td>
<td>.887</td>
<td>1</td>
<td>−2.72 −1.96</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Oxaloacetate</td>
<td>−3.64</td>
<td>1.092</td>
<td>.005</td>
<td>−6.52 −.76</td>
</tr>
<tr>
<td>Control</td>
<td>Oxaloacetate</td>
<td>−2.89</td>
<td>1.039</td>
<td>.032</td>
<td>−5.63 −1.15</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>Pyruvate</td>
<td>.38</td>
<td>.887</td>
<td>1</td>
<td>−1.96 2.72</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Pyruvate</td>
<td>−3.26</td>
<td>1.084</td>
<td>.016</td>
<td>−6.12 −4.0</td>
</tr>
<tr>
<td>Control</td>
<td>Pyruvate</td>
<td>−2.51</td>
<td>1.030</td>
<td>.089</td>
<td>−5.23 .21</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Pyruvate</td>
<td>3.64</td>
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<td>3.26</td>
<td>1.084</td>
<td>.016</td>
<td>.40 6.12</td>
</tr>
<tr>
<td>Control</td>
<td>Oxaloacetate</td>
<td>.75</td>
<td>1.212</td>
<td>1</td>
<td>−2.44 3.95</td>
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<tr>
<td>Oxaloacetate</td>
<td>Glutamate</td>
<td>2.89</td>
<td>1.039</td>
<td>.032</td>
<td>.15 5.63</td>
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<tr>
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<td>−.21 5.23</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glutamate</td>
<td>−.75</td>
<td>1.212</td>
<td>1</td>
<td>−3.95 2.44</td>
</tr>
</tbody>
</table>

A generalized linear model for Neurological Severity Score (NSS). The analysis was performed using a generalized estimation equation model. Included was the NSS at 1, 24, and 48 h and 1, 2, 3, and 4 weeks. Overall, there was no significant neurologic improvement in the pyruvate group (lower NSS) when compared with control ($P < 0.05$). When compared with the glutamate group, both the pyruvate and oxaloacetate groups had improved NSS ($P < 0.02$).
regions. Damage to the CA3 region has been linked to cognitive deficits, and ischemic damage to the CA1 region has been associated with learning and memory deficits. Accordingly, systematic anatomic analyses of the hippocampus after TBI must include additional hippocampal subregions to evaluate the extent of pathology caused by injury.27,28

The short-term neurologic outcomes in treated rats are consistent with our previous findings.21,24 These studies, which were designed and powered to focus on outcomes, demonstrated a correlation between oxaloacetate treatment and the transient decrease of blood glutamate concentrations, thus establishing its scavenging properties.21 By blocking the biochemical pathways involved in the transformation of glutamate into 2-ketoglutarate, a reversal of the effects attributed to oxaloacetate was shown, thus establishing the role of oxaloacetate as a glutamate scavenger, rather than as an agent acting directly on the brain.21,24 The correlation between the number of surviving neurons in the hippocampus, oxaloacetate treatment, and a decrease in blood glutamate concentration indicates that the impact of oxaloacetate on the brain is most likely the result of its brain glutamate scavenging properties. One could argue that this effect may be attributable to the direct actions of oxaloacetate on brain hippocampal neurons. The tight synergism between blood oxaloacetate and glutamate-oxaloacetate transaminase make the possibility of a brain intraparenchymatic site of action unlikely because of the kinetics of entry into the brain (oxaloacetate is dependent on a dicarboxylate transporter, and glutamate-oxaloacetate transaminase is impermeable to an intact blood–brain barrier).

Two putative neuroprotective mechanisms could be involved and are described elsewhere.30 However, as an inhibitor of succinate dehydrogenase, oxaloacetate, if it reached brain parenchyma, should cause pseudohypoxia and the activation of hypoxia-inducible factor under normoxia.31 Hypoxia-inducible factor, which is associated with brain hypoxia and activated after TBI, will cause the up-regulation of aquaporin 4, leading to brain edema.22 Thus, one would expect that oxaloacetate should contribute to brain edema formation after TBI, contrary to recent observations.22

The actions of pyruvate as a blood glutamate scavenger were found to be similar to those of oxaloacetate.22 Altogether, these findings support the idea that neuroprotection by pyruvate and oxaloacetate is the result of their blood glutamate scavenging activity, which is consistent with previous findings.17,20–22,24

In the glutamate treatment group, the artificial elevation of blood glutamate led to significant neuronal loss in all hippocampus regions compared with sham, control, and treatment groups. This feature may be a result of the inability to remove excess glutamate from the brain’s ECF, leading to excitotoxicity and its various sequel: necrosis, apoptosis, and neuronal loss.15 The glutamate treatment group demonstrates worse neurologic outcomes than do the oxaloacetate and pyruvate groups throughout the 4 weeks after injury. Nevertheless, in comparison with the control group outcomes, the 10-fold increase in blood glutamate concentration in the glutamate treatment group does not lead to an inferior neurologic outcome of the same magnitude and remains statistically insignificant. Previous studies, focused on the correlation between blood glutamate concentrations and neurologic improvement, reached similar conclusions.17,20,21,24 Blood glutamate concentration fluctuations were found to be self-limiting and presumably are

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Fig. 3. Number of surviving neurons in five different regions of the hippocampus 30 days after traumatic brain injury. Data are presented as mean ± SD. The number of surviving neurons in the treatment groups is increased compared with the control or glutamate groups.

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part of a complex counter-regulatory mechanism, that may be catalyzed by the artificial increase of blood glutamate. Exogenous glutamate introduced into the circulation may be metabolized or rapidly distributed to the body compartments, thus accounting for the limited effect on neurologic outcomes.

One major limitation in our rat model of TBI is that outcome is assessed by the NSS, which is mainly a motor test. As such, the test may not be sensitive enough to detect behavioral and cognitive changes found in patients with TBI in the long term. The long-term morphologic changes after TBI may account for some of these changes. In addition, the significant recovery and neurologic improvement within the first 48 h after TBI may have clinical importance, despite that the long-term outcome seems unchanged. Another possible explanation is based on glutamate, in normal concentrations, being critical for preserving normal central nervous system function and maintaining the integrity between neurons. We speculate that the artificial elevation of plasma glutamate concentration may partially decrease the rate of elimination of excess glutamate from the brain fluids into blood but also may enhance the formation of new synaptic pathways between surviving neurons, thus limiting the loss of neuronal integrity.

Several closed-head–injury models are used and have been reviewed extensively: fluid percussion, rigid indentation, inertial acceleration, impact acceleration, and weight drop. The weight-drop closed-head–injury model used in our study has several advantages and is close to clinical situations. Rodent studies using this model

Fig. 4. CA1-CA4 and dentate gyrus (DG) regions of the hippocampus and their location in the hippocampus. A comparison of treatment, control, glutamate, and sham groups: The effect of glutamate and oxaloacetate is reflected in histologic slices of hippocampus. The morphology of hippocampus is preserved in all groups. DG remains least affected in comparison of control (A, B) and treatment groups (C, D). Sham group (no traumatic brain injury [TBI]) (A). Oxaloacetate treatment group (B). Cellularity is nearly preserved with no significant pathologic changes. The CA4 region demonstrates a slight decrease in number of cells. Control group (TBI, no treatment) demonstrates a significant loss of cellularity in all regions aside from DG (C). Glutamate treatment: Greater loss of cellularity in all regions aside from DG (D). Magnified CA2 region shows greater loss of cellularity in control (E) versus oxaloacetate (F). Irregular, hyperchromatic nuclei are suggestive of apoptotic cells.
demonstrated short-term neurologic impairment and neuronal loss in cortex and more distantly in the hippocampus.\textsuperscript{46–48} The absence of trephination and the vertex rodent skull led to wide variability in the extent of damage and subsequently the results, particularly compared with the fluid percussion model, which produces a focused reproducible lesion. Moreover, long-term investigations have found long-term neurologic dysfunction when using the latter model.\textsuperscript{49}

The applicability of treatment with blood glutamate scavengers in human subjects remains a major concern. Regrettably, no pharmacokinetic, pharmacodynamic, or safety studies have been performed with oxaloacetate, thus limiting the possibility of suggesting this treatment in humans. Nevertheless, data accumulated to date have not indicated the existence of any serious adverse effects, and the accumulating evidence of its beneficial effect in brain injury warrants the design of such studies. Several human studies have shown that brain glutamate concentrations remain increased for longer periods of time.\textsuperscript{7,8,50} The relatively short-lived, glutamate-reducing effect of oxaloacetate and pyruvate in a rat model of TBI,\textsuperscript{21,22} which is tightly linked to the limited duration of excess glutamate in the rat brain after TBI, thus maintaining a correlation between increased brain ECF glu-

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**Fig. 5.** High-power micrographs of CA2 hippocampal region of two typical treatment and glutamate treatment groups. Nearly normal cellularity of the oxaloacetate-treated group (A). The glutamate-treated group demonstrated a significant loss of cellularity (B). The irregular, hyperchromatic nuclei are suggestive of apoptotic cells. TBI = traumatic brain injury.

**Table 4.** Primary Outcomes Comparison of Brain Cell Viability

<table>
<thead>
<tr>
<th>Group (I)</th>
<th>Group (J)</th>
<th>Mean Difference (I-J)</th>
<th>P Value (Bonferroni)</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>Pyruvate</td>
<td>38.50</td>
<td>.001</td>
<td>10.35</td>
<td>66.65</td>
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<td></td>
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<td>&lt;.001</td>
<td>26.85</td>
<td>45.31</td>
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<td>Glutamate</td>
<td>133.10</td>
<td>&lt;.001</td>
<td>122.23</td>
<td>143.97</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>80.40</td>
<td>&lt;.001</td>
<td>68.52</td>
<td>92.28</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Oxaloacetate</td>
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<td>1.000</td>
<td>−31.47</td>
<td>26.63</td>
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<tr>
<td></td>
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<td>94.60</td>
<td>&lt;.001</td>
<td>64.99</td>
<td>124.21</td>
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<tr>
<td>Oxaloacetate</td>
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<td>&lt;.001</td>
<td>84.01</td>
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<tr>
<td></td>
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<td>&lt;.001</td>
<td>30.46</td>
<td>58.18</td>
</tr>
<tr>
<td>Control</td>
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<td>&lt;.001</td>
<td>−92.28</td>
<td>−68.52</td>
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<td></td>
<td>Oxaloacetate</td>
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<td>&lt;.001</td>
<td>−58.18</td>
<td>−30.46</td>
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<tr>
<td></td>
<td>Glutamate</td>
<td>52.70</td>
<td>&lt;.001</td>
<td>37.69</td>
<td>67.71</td>
</tr>
</tbody>
</table>

Primary outcomes comparison of brain cell viability across all hippocampal regions. The analysis was performed using a generalized estimation equation model. Bonferroni correction to the group number was performed. Significant differences exist between treatment groups (pyruvate and oxaloacetate) and control, and between control + treatment groups (pyruvate and oxaloacetate) and glutamate.
Glutamate and decreased blood glutamate\textsuperscript{8,51} may well be maintained in humans, resolving the apparent temporal discrepancy. Because the reducing effect of oxaloacetate and pyruvate has not been evaluated on humans, we speculate that this effect may be longer lasting, correlating to the increased brain ECF glutamate concentration. Such a regulatory system may take part in maintaining glutamate homeostasis, eliminating excess glutamate without preventing glutamate from performing its role in neuronal signaling and repair.\textsuperscript{40}

This study deepens our knowledge and understanding of glutamate-induced neurotoxicity and blood glutamate scavenger-induced neuroprotection. The effect of blood glutamate scavengers on rats with TBI may unveil new therapeutic possibilities applicable in a variety of clinical settings. Blood glutamate scavenging represents a fundamentally different approach than does administration of glutamate receptor antagonists, which ought to gain access to the brain parenchyma. The scavengers eliminate only pathologically increased concentrations of glutamate in brain fluids, which at least in rat, are relatively short lived. This process slows and eventually halts when the excess glutamate concentrations have decreased to concentrations less than the threshold of activation of the brain’s endothelial glutamate transporters \textit{(i.e., less than their Km values)}, which mediate the glutamate efflux into the blood.

In summary, this study demonstrates that the blood glutamate scavengers oxaloacetate and pyruvate provide neuroprotection after TBI, expressed by reduced neuronal loss in the hippocampus and improved neurologic outcomes in the acute phase, which are associated with reduced blood glutamate concentrations.

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