Reduction of brain edema and expression of aquaporins with acute ethanol treatment after traumatic brain injury

Laboratory investigation

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Object. Previous studies have demonstrated that traumatic brain injury (TBI) causes brain edema by allowing excessive water passage through aquaporin (AQP) proteins. To establish the potential neuroprotective properties of ethanol as a post-TBI therapy, in the present study the authors determined the effect of ethanol on brain edema, AQP expression, and functional outcomes in a post-TBI setting.

Methods. Adult male Sprague-Dawley rats weighing between 425 and 475 g received a closed head TBI in which Maramarou’s impact-acceleration method was used. Animals were given a subsequent intraperitoneal injection of 0.5 g/kg or 1.5 g/kg ethanol at 60 minutes post-TBI and were killed 24 hours after TBI. Brains were subsequently examined for edema along with AQP mRNA and protein expression. Additional animals treated with either 0.5 g/kg or 1.5 g/kg ethanol at 60 minutes post-TBI were designated for cognitive and motor testing for 3 weeks.

Results. Ethanol administration post-TBI led to significantly (p < 0.05) lower levels of brain edema as measured by brain water content. This downregulation in brain edema was associated with significantly (p < 0.05) reduced levels of AQP mRNA and protein expression as compared with TBI without treatment. These findings concur with cognitive studies in which ethanol-treated animals exhibited significantly (p < 0.05) faster radial maze completion times. Motor behavioral testing additionally demonstrated significant (p < 0.05) beneficial effects of ethanol, with treated animals displaying improved motor coordination when compared with untreated animals.

Conclusions. The present findings suggest that acute ethanol administration after a TBI decreases AQP expression, which may lead to reduced cerebral edema. Ethanol-treated animals additionally showed improved cognitive and motor outcomes compared with untreated animals.

Key Words • blood-brain barrier • cognitive behavior • rat • motor behavior • ethanol • neuroprotection • traumatic brain injury

Each year in the US, 2 million people sustain TBIs, leading to an estimated 56,000 deaths and 80,000 impaired individuals, at an estimated cost of 56 billion dollars.32 Although TBI remains a significant health problem, clinical outcomes from many post-TBI therapeutic interventions have been disappointing.34 One of the more serious consequences of TBI is brain edema, which in itself leads to a wide array of deleterious effects. The expansion of brain volume leads to increased intracranial pressure, which can subsequently cause sequelae such as secondary cell injury, brain herniation, coma, and failure of the respiratory and/or cardiovascular systems.24 Despite the fact that cerebral edema has long been recognized as a critical post-TBI issue, treatment options for this condition remain obsolete and of limited efficacy.20 Recent research has elucidated the role of AQPs in inducing brain edema. Aquaporins are water-selective, plasma membrane channels that increase water permeability in cells.20,29 Of the ones we investigated, AQP4 is primarily expressed in astrocytes and ependymal cells.

Abbreviations used in this paper: AQP = aquaporin; BBB = blood-brain barrier; PCR = polymerase chain reaction; TBI = traumatic brain injury.
Traumatic brain injury, BBB disruption, and brain edema

both of which play a critical role in maintenance of the BBB;\(^{23}\) AQP9 has also been shown to be expressed in the BBB.\(^{11}\) Alteration in the levels of either AQP could conceivably lead to increased permeability of the BBB and induction of cerebral edema. In that regard, we and others have recently reported increases in both AQP4 and AQP9 levels post-TBI.\(^{20}\)

Although the pathophysiological role of AQPs in a post-TBI setting remains to be elucidated, regulation of AQP production in pathological states is emerging as a putative therapeutic target to improve neurological outcomes. One such attempt may involve acute administration of ethanol after TBI. Whereas some studies have reported worse clinical outcomes in patients with TBI,\(^{2,22}\) paradoxically, other clinical studies have reported improved outcomes in patients with higher blood alcohol content.\(^{23,27}\) Although ethanol has been known to have neuroprotective properties in TBI,\(^{2}\) its use as a therapy after the onset of TBI has never been investigated. Our previous studies in stroke models revealed neuroprotective properties of ethanol when used as a postsischemia treatment; ethanol was found to reduce AQP and matrix metalloproteinase expression and brain edema,\(^{28}\) as well as behavioral dysfunction.\(^{31}\) Although the aforementioned studies support the notion of ethanol-induced neuroprotection, the use of ethanol as a therapy post-TBI and the mechanism behind its protection remain to be elucidated.

The present study’s aim was to investigate whether the neuroprotective effects of ethanol may be causally associated with alterations of AQP levels induced by brain trauma. More specifically, its aim was to determine whether acute ethanol administration post-TBI can lead to decreased AQP expression, together with decreased cerebral edema levels and improved functional outcomes. The current study poses the following questions. 1) Does post-TBI ethanol administration help maintain BBB integrity? 2) Can the upregulation of AQPs in a post-TBI setting be ameliorated by ethanol administration? 3) Does ethanol treatment improve functional outcomes?

**Methods**

**Experimental Animals**

A total of 46 adult male Sprague-Dawley rats (Charles River) weighing between 425 and 475 g were used. For edema, AQP mRNA, and protein studies, rats were randomly assigned to one of 4 groups: 1) sham injured, untreated (5 rats); 2) 0.5 g/kg ethanol (6 rats); 3) 1.5 g/kg ethanol (6 rats); and 4) TBI-only group (5 rats). Animals were killed 24 hours posttreatment. For cognitive and motor testing, rats were randomly assigned to one of 4 groups: 1) sham injured, untreated; 2) 0.5 g/kg ethanol; 3) 1.5 g/kg ethanol; and 4) TBI-only group (6 rats per group). Animals used for cognitive and motor testing were tested up to 3 weeks post-TBI. All ethanol-treated animals received this substance 60 minutes post-TBI by intraperitoneal injection.

**Closed Head Trauma Model and Ethanol Treatment**

A modified Marmarou TBI model was used.\(^{18}\) This method reproduces a closed head TBI, which unlike an open head TBI, is more likely to be seen in human patients. Rats were anesthetized with halothane and a midline incision was then made, exposing the periosteum covering the vertex of the skull. A metal disc was next placed on the skull between the coronal and lambdoid sutures. Rats were placed on a foam cushion with their head secured to prevent movement; they were then positioned under a plastic tube, through which a weight of 450 g was dropped from a height of 2 m onto the metal disc. Animals were then returned to their respective cages and allowed to move freely while recuperating from anesthesia. Animals received either a 0.5-g/kg or a 1.5-g/kg dose of ethanol via an intraperitoneal injection 60 minutes post-TBI, depending on their group assignment. Sham-injured, untreated animals were subjected to the exact same surgical procedure as the other animal groups, without the actual dropping of the weight or the ethanol administration. Following surgery, body temperature was monitored using a rectal thermometer, and maintained at 37°C by using a Deltaphase thermal pad (Braintree Scientific, Inc.) for 30 minutes post-TBI.

**Determination of Brain Edema as Measured With Brain Water Content**

Integrity of the BBB after TBI was determined by brain edema, which was quantified by brain water content. Brains were first weighed to determine their wet weight, and then placed into a 72°C thermal oven for 72 hours. Brain tissue was then weighed again to assess dry weight. The formula (wet weight – dry weight)/wet weight × 100% was used to calculate the water content percentage of each brain.\(^{12}\)

**Gene Expression of AQP4 and AQP9**

Whole-brain samples of each animal were homogenized and prepared for both mRNA and protein processing. A sensitive real-time reverse transcription PCR technique\(^{2}\) was used to determine the expression of target genes. The total RNA from half the amount of samples containing whole brain was isolated using an RNA STAT-60 kit, according to the manufacturer’s instructions (Invitrogen). The cDNA was then amplified using an Eppendorf Real-Time PCR Thermocycler for real-time PCR, with SYBR Green PCR Master Mix (Eppendorf). The gene-specific rat primers for AQP4 and AQP9 are shown in Table 1. For internal PCR control, the rat ribosomal protein L32 (rpL32) gene, a housekeeping gene, was used. Reactions were performed in a 20-μl volume with 0.5 μM

**TABLE 1: Sequences of primers for AQP4 and AQP9 mRNA analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP4</td>
<td>primer (forward)</td>
<td>5′-TTGGACCAATCATAGGCGGC-3′</td>
</tr>
<tr>
<td></td>
<td>primer (reverse)</td>
<td>5′-GGTCAATGTGACATCGATGC-3′</td>
</tr>
<tr>
<td>AQP9</td>
<td>primer (forward)</td>
<td>5′-GATGCCTCTGAGAAGGACG-3′</td>
</tr>
<tr>
<td></td>
<td>primer (reverse)</td>
<td>5′-AGAGACCTACGACTGCG-3′</td>
</tr>
<tr>
<td>rpL32</td>
<td>primer (forward)</td>
<td>5′-TGTCCTAAGAGCGAGCAG-3′</td>
</tr>
<tr>
<td></td>
<td>primer (reverse)</td>
<td>5′-CGTTGAGGATGTGACTGTA-3′</td>
</tr>
</tbody>
</table>
of primers. The PCR cycles consisted of an initial denaturation step at 95°C for 9 minutes, followed by 45 cycles of a 95°C denaturation for 10 seconds, 60°C annealing for 5 seconds, and 72°C extension for 15 seconds. The relative mRNA levels of gene expression were determined using the threshold cycle (Ct) and arithmetic formulas. The expression of the target genes (AQP4 and AQP9) from the experimental groups therefore represented the fold-difference expression relative to the reference gene.

**Determination of AQP4 and AQP9 Protein Expression Using Western Blot Test**

Cerebral samples were processed in a lysis buffer with protease inhibitors. All samples were normalized using the Bradford assay (Bio-Rad) to ensure equal protein concentrations in each sample. Ten-microliter samples were then mixed with a sodium dodecyl sulfate buffer. Samples were next run through a 13% polyacrylamide gel (Bio-Rad) and then transferred to a nitrocellulose membrane (Bio-Rad). This experiment used 2 different primary antibodies: polyclonal rabbit anti-AQP4 (1:500, Santa Cruz), and polyclonal anti-AQP9 (1:2000, Santa Cruz). A polyclonal anti-β-actin antibody (1:1000, Santa Cruz) was also used to ensure equal protein loading. Membranes were incubated with the primary antibody at 4°C for 24 hours. On completion of primary antibody incubation, membranes were washed 3 times and then incubated at room temperature with a secondary antibody conjugated to horseradish peroxidase (Sigma) for 1 hour. The targeted antigens (AQP4 and AQP9) were visualized using chemical luminescence techniques (ECL, Amersham Pharmacia Biotech). An image analysis program (ImageJ 1.42, NIH) was used to determine the amounts of protein expression in the control and experimental groups.

**Behavior Testing**

To assess cognitive outcomes the radial arm maze was used, in which animals were tested twice a day for 18 consecutive days, beginning at 24 hours post-TBI. Bait (Froot Loops cereal, Kelloggs Co.) was placed in 4 different arms (arms 1, 2, 4, and 7). Each rat being tested was placed in the center of the radial maze and given a maximum of 10 minutes to find all 4 baits. If the animal was unable to find all 4 baits within the allotted 10 minutes, its time of maze completion was marked as 10 minutes.

The foot-fault placing test was used to determine motor coordination. Each rat underwent testing at 1, 3, 5, 7, 10, 14, and 21 days post-TBI. For foot-fault testing, a steel girder in the pattern of a checkerboard was used, with holes measuring 2 x 2 cm. Animals were placed on the girder and given a total of 2 minutes of walking time. An error was recorded each time a rat’s foot fell through a hole.

**Statistical Analysis**

Statistical analysis was performed using SPSS for Windows, version 13.0 (SPSS, Inc.). Differences among groups were assessed using 1-way ANOVA, with significance being set to p < 0.05. Post hoc analysis was additionally performed to identify which groups differed from each other.

**Results**

**Brain Edema**

As shown in Fig. 1, ANOVA and post hoc analysis indicated that post-TBI ethanol administration led to decreased edema levels when compared with rats in the TBI-only group (F[2,14] = 36.16, p < 0.01). Both ethanol treatment groups demonstrated significantly decreased brain water content compared with the TBI-only group. Post hoc analysis indicated no significant difference between the ethanol treatment groups (0.5 g/kg vs 1.5 g/kg).

**Aquaporin Expression**

Real-time PCR data showed that ethanol given post-TBI significantly decreased mRNA levels of both AQP4 (F[2,14] = 11.49, p < 0.001) and AQP9 (F[2,14] = 51.57, p < 0.001) (Fig. 2). Significant decreases in AQP mRNA were seen with both the 0.5-g/kg and 1.5-g/kg treatment groups. Post hoc analysis failed to show a significant difference in the mRNA reduction between the 2 ethanol doses (0.5 g/kg vs 1.5 g/kg). Results with AQP protein expression demonstrated that post-TBI ethanol administration led to a significant downregulation of protein expression of AQP4 (F[2,14] = 4.541, p < 0.05) and AQP9 (F[2,14] = 34.055, p < 0.01) (Fig. 3). However, unlike what was seen with the mRNA results, post hoc analysis indicated that downregulation of AQP4 protein expression was only seen with the 1.5-g/kg dose (upper panels of Fig. 3). Although the 0.5-g/kg ethanol treatment group did display decreased AQP4 protein expression compared with the TBI-only group, it did not represent a significant decrease. No significant difference between the 2 ethanol treatment groups was seen in terms of AQP9 protein expression, with both treatment groups producing significant AQP9 protein downregulation (lower panels of Fig. 3).

**Cognitive and Motor Functions**

Immediately following ethanol administration, animals displayed no grossly observable clinical side effects. Cognitive performance studies indicated an ethanol-induced neuroprotection. Ethanol-treated animals demonstrated significantly faster maze completion times throughout the trial (F[3,15] = 13.362, p < 0.01) (Fig. 4) compared with TBI-only animals. The TBI-only animals displayed maze completion times of 10 minutes up until Day 7, whereas both ethanol-treated groups were exhibiting maze completion time improvements within the first few days of the trial. Although ethanol-treated animals showed significant improvements in maze completion time, post hoc analysis did not show a significant difference between the 0.5-g/kg and 1.5-g/kg ethanol treatment groups.

Regarding motor testing, foot-fault placing revealed significantly fewer faults committed by ethanol-treated rats than animals in the TBI-only group (F[3,15] = 19.15, p < 0.01) (Fig. 5). This neuroprotective effect was only seen with the higher dose, 1.5 g/kg ethanol. Post hoc analysis indicated that ethanol-induced neuroprotection was not seen with the 1.5-g/kg treatment group until Day 5, whereas the 0.5-g/kg treatment group showed no significant improvements during the trial.

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Discussion

In our study, ethanol given shortly after TBI, especially at a dose of 1.5 g/kg, resulted in reduced cerebral edema, which was temporally associated with decreased AQP4 and AQP9 mRNA and protein expression. Ethanol treatment also improved cognitive outcomes in both groups. However, significant motor improvements were only seen in the 1.5-g/kg group. These findings support the notion that ethanol, especially at a higher dose, has neuroprotective properties when given acutely after TBI. For comparison, prior studies have indicated that 0.5-g/kg and 1.5-g/kg doses of ethanol produce human blood ethanol levels of 40 mg/dl and 89 mg/dl, respectively, the latter of which is within the legally intoxicated range of 80–100 mg/dl for driving.

Although ethanol’s mechanism for neuroprotection remains largely conjectural, studies in the literature suggest the following mechanisms. Glutamate, an excitatory neurotransmitter, has been found to increase in concentration following head trauma. Several studies have also shown that ethanol, when administered acutely, suppresses glutamate receptor function, particularly the N-methyl-D-aspartate receptor. A previous study in which acute ethanol intoxication was induced 2 hours prior to trauma suggests that neuroprotection may be due to N-methyl-D-aspartate receptor inhibition. Alternatively, pre-TBI eth-
anol treatment–induced neuroprotection could be due to the following mechanisms: 1) dampening of the sympathetic response;\(^3^3\), 2) decrease in the amount of inflammatory cytokines;\(^9\), and/or 3) increase in vasodilation, which would counter the vasoconstriction and decreased blood flow effects observed after brain trauma.\(^1^3\)

The results of this study, for the first time to our knowledge, suggest a mechanism behind ethanol’s neuroprotective properties—reduction of cerebral edema—seen with post-TBI ethanol treatment. Administration of ethanol may serve to ameliorate many of the deleterious effects of cerebral edema, such as increased intracranial pressure, decreased perfusion, decreased oxygenation, potential herniation, and respiratory depression.\(^2^9\) Limiting the effects of the aforementioned outcomes would serve to limit brain injury as well as decrease morbidity and mortality after TBI.

In our study, decreased edema levels were associated with a reduction in AQP4 and AQP9 expression. Recent research in the area of cerebral edema has centered on AQPs, which promote the diffusion of water across the plasma membrane.\(^1^1\) Upregulation of AQP4 has also been shown to contribute to the formation of cytotoxic edema in numerous pathological brain conditions, including ischemia, neoplasms, and the topic of this investigation—trauma.\(^1^5,1^7,2^6,3^0\) Other studies further indicate that AQP9 is upregulated following cerebral insults, and participates in edema formation.\(^2\) Conversely, reduced AQP4 and/or AQP9 could result in less water transport through the BBB, which may decrease the amount of edema. This was in fact confirmed by the observations in the present study, in which post-TBI ethanol treatment resulted
in reduced AQP expression and amelioration of the TBI-induced cerebral edema. The decreased edema levels and reduced AQP expression were also associated with overall improved functional outcomes, as evidenced by faster maze completion times and fewer foot faults. Although the decrease in AQP expression was seen in concert with ethanol administration, there remains a possibility that the change in AQP expression is an epiphenomenon. Therefore, further work in which transgenic rodent models with varying levels of AQP expression are used is of interest.

Although the results of this study point toward ethanol-induced neuroprotection, it is likely that this neuroprotection may only occur at certain doses. A previous study demonstrated that whereas doses of 3 g/kg ethanol led to increased mortality, lower doses of 1.5 g/kg ethanol were able to improve motor outcomes in experimental animals when compared with animals with no ethanol treatment. Accordingly, the highest dose (1.5 g/kg) found not to be associated with adverse outcomes was chosen in the present study. In addition, the dose of 0.5 g/kg ethanol was also chosen because studies have already established neuroprotective effects with 1 g/kg, thus this study sought to determine if neuroprotection would also be observed at lower doses. Although prior work advocates the notion of ethanol-induced neuroprotection, many of these conclusions are based on the premise of pretrauma ethanol administration. Although such work has done much to broaden the understanding of ethanol-induced neuroprotection, it does not reveal as much regarding therapeutic options for TBI. Administering ethanol in a pre-TBI setting does not represent a feasible or sensible option for treating TBIs. As a result, this study chose to examine the effects of ethanol when given post-TBI, specifically at 60 minutes post-TBI. Regarding the route of ethanol administration, intraperitoneal injection was chosen due to the ease of administration. In the clinical setting, intravenous administration of ethanol would probably be the preferred method of administration. We hypothesize that intravenous or intraarterial ethanol administration would produce similar neuroprotective effects as intraperitoneal administration. However, this is an area that requires further investigation.


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