Hypertonic Saline Alleviates Brain Edema After Traumatic Brain Injury via Downregulation of Aquaporin 4 in Rats

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Background:
Hypertonic saline (HS) has been successfully used for treatment of various forms of brain edema. Decreased expression of aquaporin (AQP)4 and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been linked to edema pathogenesis. This study examined the effect of 3% HS on brain edema in a rat model of traumatic brain injury (TBI).

Material/Methods:
Sprague-Dawley rats were subjected to TBI induced by a controlled cortical impactor. The HS group was injected with 3% NaCl until the end of the study period. AQP4, TNF-α, IL-1β, and caspase-3 levels were measured by Western blotting, immunohistochemistry, enzyme-linked immunosorbent assay, and quantitative real-time PCR. Brain water content was also measured. Apoptotic cells in brain tissue were detected with terminal deoxynucleotidyl transferase dUTP nick-end labeling. Brain water content decreased following treatment with 3% HS relative to the TBI group.

Results:
This was accompanied by decreases in AQP4, TNF-α, and IL-1β mRNA and protein levels. TBI resulted in increases in caspase-3 mRNA expression and the number of apoptotic cells; treatment with 3% HS suppressed apoptosis as compared to the TBI group.

Conclusions:
Treatment with 3% HS ameliorated TBI-induced brain edema, possibly by suppressing brain edema, pro-inflammatory cytokine expression, and apoptosis.

MeSH Keywords: Apoptosis Inducing Factor • Aquaporin 4 • Brain Edema • Saline Solution, Hypertonic
ANIMAL STUDY

Background

Traumatic brain injury (TBI) can result in extensive physical, behavioral, and cognitive deficits [1–3]. Primary injury occurs simultaneously with TBI impact, leading to death or disability through disruption of the blood brain barrier and vasculature contributing to edema [4]. This is followed by secondary injury involving activation of microglia and astrocytes in the brain, which produce cytokines and chemokines, and recruitment of peripheral immune cells to the brain [5].

The aquaporin (AQP) family of water-channel proteins regulates water homeostasis [6]. To date, 13 conserved members have been identified in mammals; AQP3, AQP4, AQP5, AQP8, and AQP9 are expressed in cultured rat astrocytes [7]. AQP4 plays a key role in cerebral volume regulation following trauma, inflammation, and ischemia, as well as in tumors and metabolic disorders [8,9]. Several studies have shown that AQP4 modulates memory and synaptic plasticity via regulation of glutamate transporter expression and contributes to the development of brain edema [10–13].

Hyperosmolar therapy with hypertonic saline (HS) solutions is a commonly used treatment for cerebral edema caused by cerebral ischemia, cerebral hemorrhage, or TBI [14,15]. HS was shown to be more effective in reducing brain edema as compared to ischemia, cerebral hemorrhage, or TBI [14,15]. HS was shown to be more effective in reducing brain edema as compared to ischemia, cerebral hemorrhage, or TBI [14,15]. HS was shown to be more effective in reducing brain edema as compared to ischemia, cerebral hemorrhage, or TBI [14,15]. HS was shown to be more effective in reducing brain edema as compared to ischemia, cerebral hemorrhage, or TBI [14,15].

To test this hypothesis, we investigated the effects of HS infusion on cerebral water content, inflammation, and apoptosis in a rat model of TBI. Our results indicate that HS treatment alleviates TBI-induced brain edema by inhibiting the expression of factors related to brain water regulation, inflammation, and apoptosis.

Material and Methods

Animals

Animal procedures were performed at the Animal Experiment Center of Zhejiang Chinese Medicine University. Experiments were performed on male Sprague-Dawley rats (250–300 g, 3–4 months old) that were group-housed in a temperature- and humidity-controlled environment (22±1°C and 55±%, respectively), had free access to food and water, and were maintained on a 12:12-h light/dark cycle.

TBI model

TBI was induced in rats using a controlled cortical impactor (TBI-0310 Head Impactor; Precision Systems and Instrumentation, USA) with a 3-mm diameter metal tip [18]. Rats were anesthetized through intraperitoneal injection of 3.6% chloral hydrate (4 g/kg) and then fastened in a stereotactic fixing frame. Rectal temperature was monitored and body temperature was maintained at approximately 37°C by placing the animals on an electric heating blanket [19]. A 3-mm diameter hole was made in the cranium using an electric hand drill after exposing the skull. The right hemisphere was subjected to contusion injury at a speed of 3.0 m/s and depth of 2 mm [20–24]. The scalp was sterilized and sutured shut after the impact. The sham group rats (n=36) underwent craniotomy but were not subjected to impact injury. After 6 h, rats in the TBI and sham groups were injected with normal saline via the caudal vein; another group of rats (n=36) were injected with 3% NaCl (3% HS). Animals were sampled at 0, 6, 24, and 48 h.

Brain water content

Rats were sacrificed and brain tissue was removed and immediately placed in Petri dishes containing saline-moistened filter paper to prevent water evaporation. Blood was blotted with a filter paper and the cerebellum, brainstem, and pia were removed. The remaining brain tissue was weighed to determine wet weight within 5 min of brain removal. The brain tissue was weighted immediately after removal (wet weight) and again after drying in an oven at 100°C for 48 h. Water content was calculated according to the formula: ([wet weight]−[dry weight])/(wet weight)×100%.

Quantitative real-time (qRT)-PCR

Total RNA was extracted from the rat brain using an RNeasy Lipid Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. cDNA was prepared from 1 μg total RNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) and used for qRT-PCR, which was performed on a CFX96 system (Bio-Rad, Hercules, CA, USA) using primers and SYBR Green mix (Takara Bio, Otsu, Japan). Amplification was performed over 45 cycles of 95°C for 15 s and 60°C for 30 s after holding at 68°C for 15 s and 95°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Relative expression levels were determined with the cycle threshold method. Primer sequences are listed in Table 1.

Western blotting

AQP4 levels in brain tissue at 0, 6, 24, and 48 h were determined by Western blotting. Brain tissue was homogenized and diluted to a concentration of 1 μg/μl in PBS. A 20 μl volume of brain homogenate was loaded to each well of an 8% mini SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and incubated with primary antibodies at 4°C overnight. After washing with Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with secondary antibodies at room temperature for 1 h. After washing, protein bands were visualized using an enhanced chemiluminescence western blotting detection system.
Table 1. Primer sequences.

| Primer   | Forward (5’-3’)           | Reverse (5’-3’)          |
|----------|---------------------------|--------------------------|
| IL-1β    | GCTTCAGCGAGGCAGATATCA    | TGCAGTTGTCTAATGGGAACG    |
| TNF-α    | TACTGAACTTCCGGGTTGATCG   | CCACCTGGTGTTGGCTACG      |
| Caspase 3| AGAACCTGGACTCGGTTATGAG   | GAACCATGACCCGTCCCTTG     |
| GAPDH    | GTGCCAGGCTCTGTCATAG      | CTTTGTCACAAAGAAGGCAG     |

Detection of apoptotic cells with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Brain tissue was harvested and apoptotic cells were detected with a TUNEL assay kit (DeadEnd fluorometric TUNEL System; Promega, Madison, WI, USA). The number of TUNEL-positive cells was counted at 400× magnification and results from 3 sections per brain were averaged [26].

Statistical analysis

Data are expressed as mean ± standard error. Differences between groups were analyzed with SPSS v.19.0 (IBM, Armonk, NY, USA). Semi-quantitative analysis of histological staining was performed by a single investigator blinded to treatment groups; differences between groups were evaluated by the Student’s t test. For Western blotting results, differences in protein expression levels were evaluated by two-way analysis of variance (ANOVA) with repeated measures, followed by a post hoc test. Differences in brain water content and qRT-PCR results were assessed with one-way ANOVA followed by a post hoc test. P values <0.05 were considered statistically significant.

Results

HS treatment reduces brain water content in a TBI model

Brain water content was measured to determine the extent of edema in the brain tissue of TBI rats. The brain water content was higher in TBI than in sham rats (Figure 1). Treatment with 3% HS decreased brain water content after 6, 24, and 48 h as compared to the TBI group (Figure 1).

To investigate the mechanism by which TBI increases brain water content, AQP4 protein expression in the brain was detected by Western blotting and immunohistochemistry. AQP4 level was increased in the TBI group as compared to sham animals at each time point (Figure 2A, 2B). Treatment with 3% HS inhibited AQP4 expression at 6, 24, and 48 h as compared to the TBI group (Figure 2A, 2B). Immunohistochemical detection of AQP4 distribution in brain tissue showed similar

in lysis buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors on ice. Proteins were separated by polyacrylamide gel electrophoresis and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) that was probed with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized with Super Signal chemiluminescent substrate (Pierce). GAPDH expression was detected with an antibody (1: 1500; Abcam, Cambridge, MA, USA) as a loading control.

Immunohistochemistry

Rats were perfused with saline solution followed by 4% paraformaldehyde after deep anesthesia with sodium pentobarbital. Their brains were removed and immediately post-fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 4-μm coronal sections that were incubated overnight at 4°C with primary antibody against AQP4 (1: 200; Abcam). After 3 washes with phosphate-buffered saline (PBS), sections were incubated for 60 min with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) at room temperature. Immunoreactivity was visualized with a BX51T-PHD-J11 fluorescence microscope (Olympus, Tokyo, Japan) and analyzed with Image-Pro Plus v.4.5 software (Media Cybernetics, Rockville, MD, USA) [25].

Analysis of TNF-α and IL-1β expression by enzyme-linked immunosorbent assay (ELISA)

The right hemisphere of the brain was dissected from rats at 0, 6, 24, and 48 h and immediately weighed. The tissue was frozen in 1 ml PBS (pH 7.4) and homogenized, centrifuged at 12 000×g at 4°C for 20 min. The protein levels of TNF-α and IL-1β were determined using ELISA kits for TNF-α (Immuno-Biological Laboratories, Hamburg, Germany; cat. no. JP27194) and IL-1β (Immuno-Biological Laboratories, Minneapolis, MN, USA; cat. no. 27193) according to the manufacturers’ protocols. Absorbance at 450 nm was measured within 30 min using an ELISA microplate reader.

ANOVA with repeated measures, followed by a post hoc test.
findings as in the Western blot analysis. AQP4 expression in the TBI group showed an increasing trend (Figure 2C), but this was abrogated by administration of 3% HS at each time point examined (Figure 2D). These results indicate that TBI induces the expression of AQP4, leading to an increase in brain water content, which was reversed by HS treatment.

**Inflammation in the TBI brain is reduced by HS treatment**

To investigate the effects of HS on TBI-induced inflammation in the brain, TNF-α and IL-1β mRNA were measured by qRT-PCR and ELISA, respectively. The levels of both factors were upregulated in the TBI group at 0, 6, 24, and 48 h relative to sham animals, but this effect was abolished following 3% HS administration (Figure 3A, 3C). The same trend was observed for TNF-α and IL-1β protein expression (Figure 3B, 3D). Thus, the increase in pro-inflammatory cytokine levels resulting from TBI is countered by HS treatment.

**Figure 1.** Brain water content following TBI and HS treatment. Brain water content was increased at 0, 6, 24, and 48 h in the TBI group relative to sham animals, which was reversed by HS treatment. * P<0.05, ** P<0.01 vs. sham group; * P<0.05, ** P<0.01 vs. TBI group.

**Figure 2.** AQP4 expression and distribution in brain tissue following TBI and HS treatment. (A) AQP4 levels at 0, 6, 24, and 48 h in the TBI and HS groups. (B) Quantitative analysis of protein expression. * P<0.05, ** P<0.01 vs. sham group; * P<0.05, ** P<0.01 vs. TBI group. (C, D) AQP4 distribution in the brain tissues of the TBI, HS, and sham groups as detected by immunohistochemistry at 0, 6, 24, and 48 h. Scale bar: 50 μm.
We also investigated whether the beneficial effects of HS treatment include inhibition of apoptosis. Caspase-3 mRNA expression was rapidly upregulated following TBI as compared to the sham group, but slowly increased at 24 and 48 h (Figure 4). Treatment with 3% HS reduced caspase-3 mRNA levels at 6, 24, and 48 h as compared to the TBI group (Figure 4). A quantitative analysis of apoptotic cells detected with the TUNEL assay indicated that the apoptosis rate was increased at 0, 6, 24, and 48 h in the TBI group as compared to sham animals (Figure 5A, 5B), although there were no significant differences between the latter 2 time points. As compared to the TBI group, administration of 3% HS reduced the rate of apoptosis at each time point.

**Discussion**

In this study, we demonstrated that TBI resulted in brain edema in rats and that this was reversed by treatment with

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**Figure 3.** TNF-α and IL-1β expression following TBI and HS treatment. (A, C) mRNA expression levels of TNF-α (A) and IL-1β (C), as determined by qRT-PCR at 0, 6, 24, and 48 h in the TBI, HS, and sham groups; GAPDH served as an internal reference. (B, D) Protein expression levels of TNF-α (B) and IL-1β (D) were measured at 0, 6, 24, and 48 h the TBI, HS, and sham groups. * P<0.05, ** P<0.01 vs. sham group; * P<0.05, ** P<0.01 vs. TBI group.

**Figure 4.** Caspase-3 mRNA expression following TBI and HS treatment. Caspase-3 mRNA level was detected by qRT-PCR at 0, 6, 24, and 48 h in the TBI, HS, and sham groups; GAPDH served as an internal reference. * P<0.05, ** P<0.01 vs. sham group; * P<0.05, ** P<0.01 vs. TBI group.

**HS treatment inhibits apoptosis following TBI**

We also investigated whether the beneficial effects of HS treatment include inhibition of apoptosis. Caspase-3 mRNA expression was rapidly upregulated following TBI as compared to the sham group, but slowly increased at 24 and 48 h (Figure 4). Treatment with 3% HS reduced caspase-3 mRNA levels at 6, 24, and 48 h as compared to the TBI group (Figure 4). A quantitative analysis of apoptotic cells detected with the TUNEL assay indicated that the apoptosis rate was increased at 0, 6, 24, and 48 h in the TBI group as compared to sham animals (Figure 5A, 5B), although there were no significant differences between the latter 2 time points. As compared to the TBI group, administration of 3% HS reduced the rate of apoptosis at each time point.
Figure 5. Detection of apoptotic cells in brain tissue following TBI and HS treatment. (A) Apoptosis rate was determined at indicated time points the TBI, HS, and sham groups. * P<0.05, ** P<0.01 vs. sham group; * P<0.05, ** P<0.01 vs. TBI group. (B) Detection of apoptotic cells with the TUNEL assay.
3% HS. TBI caused general pathological cellular and biochemical dysfunction that ultimately led to neuronal and glial cell death [27]. Osmotherapeutic agents such as HS and mannitol are currently used to treat cerebral edema and reduce intracranial pressure in ischemic stroke [28]. It was recently suggested that HS is more effective in improving cerebral edema than an equal volume of 20% mannitol [29]. We demonstrated here that brain water content continuously increased for 48 h following TBI, but that this effect was abrogated by treatment with 3% HS.

The mechanistic basis for the brain edema induced by TBI is not fully understood; however, the expression levels of the pro-inflammatory cytokines such as TNF-α and IL-1β and the complement cascade may be important factors [30]. We confirmed that TBI caused an upregulation of IL-1β and TNF-α. In this study, we found that treatment with 3% HS treatment for 6 h decreased TNF-α and IL-1β levels at 24 and 48 h. Moreover, the expression of caspase-3 – which may be activated by TNF-α via caspase-8 [31] – increased over time in the TBI group relative to sham animals, which was confirmed by the results of the TUNEL assay. However, these effects were countered by administration of 3% HS.

AQP4, the primary water-channel protein expressed in the brain, is abundant in astrocyte foot processes at the borders between the brain parenchyma and major fluid compartments, and in ependymal cells lining the ventricles containing cerebrospinal fluid [32]. AQP4 is upregulated in the ischemic brain and is thought to play an important role in the cerebrospinal fluid re- sorption and regulation of brain edema [33]. Indeed, mortality is reduced in AQP4-null mice, which are less prone to cytotoxic brain edema than their wild-type counterparts, whereas transgenic mice overexpressing AQP4 show increased brain swelling [34]. Our results show that TBI increased AQP4 protein level, which was reversed by 3% HS; this can lead to other non-osmotic effects in the treatment of brain edema. Many studies have shown that HS inhibited the expression of AQP4 in brain swelling induced by ischemic stroke and meningitis [35,36]. IL-1β induced the expression of AQP4 in microglia and astrocytes [34], and TNF-α is a key mediator of brain edema [37]. Thus, TBI induces the upregulation of AQP4 by increasing inflammation.

Conclusions

In the present study, we showed that administration of 3% HS plays a key role in the inhibition of AQP4, IL-1β, and TNF-α expression induced by TBI. Taken together, our results indicate that 3% HS is an effective treatment for suppressing inflammation and preventing neuronal damage following TBI.

Conflict of interest

None.

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