RESEARCH ARTICLE

Acute effects of human protein S administration after traumatic brain injury in mice

Xiaowei Wang[1, #], Jing Tong[2, #], Xiaodi Han[3, *], Xiaoming Qi[4], Jun Zhang[5], Erxi Wu[4, 6], Jason H. Huang[4, 6, *]

1 Center for Translational Neuromedicine, University of Rochester, Rochester, NY, USA
2 Department of Neurosurgery, 4th Affiliated Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China
3 Department of Neurosurgery, Tiantan Hospital, Beijing, China
4 Department of Neurosurgery, Baylor Scott & White Health, Temple, TX, USA
5 Department of Neurosurgery, PLA General Hospital, Beijing, China
6 College of Medicine, Texas A&M Health Science Center, Temple, TX, USA

Funding: *This work was supported in part by a University of Rochester Institutional Grant (2011NSG-Huang; to JHH), National Institute of Health (NIH-R01-NS-067435; to JHH), and the Hellen Vosberg McCrillus Plummer and Robert Edward Lee Plummer, Jr, Endowment fund from Baylor Scott & White Medical Center (to JHH).*

Abstract

Despite years of effort, no effective acute phase treatment has been discovered for traumatic brain injury. One impediment to successful drug development is entangled secondary injury pathways. Here we show that protein S, a natural multifunctional protein that regulates coagulation, inflammation, and apoptosis, is able to reduce the extent of multiple secondary injuries in traumatic brain injury, and therefore improve prognosis. Mice subjected to controlled cortical impact were treated acutely (10–15 minutes post-injury) with a single dose of either protein S (1 mg/kg) or vehicle phosphate buffered saline via intravenous injection. At 24 hours post-injury, compared to the non-treated group, the protein S treated group showed substantial improvement of edema and fine motor coordination, as well as mitigation of progressive tissue loss. Immunohistochemistry and western blot targeting caspase-3, B-cell lymphoma 2 (Bcl-2) along with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay revealed that apoptosis was suppressed in treated animals. Immunohistochemistry targeting CD11b showed limited leukocyte infiltration in the protein S-treated group. Moreover, protein S treatment increased the ipsilesional expression of aquaporin-4, which may be the underlying mechanism of its function in reducing edema. These results indicate that immediate intravenous protein S treatment after controlled cortical impact is beneficial to traumatic brain injury prognosis. Animal Use Protocols (AUPs) were approved by the University Committee on Animal Resources (UCAR) of University of Rochester Medical Center (approval No. UCAR-2008-102R) on November 12, 2013.

****Correspondence to:***
Jason H. Huang, MD, Jason.Huang@BSWHealth.org.

#These authors contributed equally to this paper.

orcid:
0000-0002-4426-0168 (Jason H. Huang)

doi: *10.4103/1673-5374.282258*

Received: *October 2, 2019*
Peer review started: *October 17, 2019*
Accepted: *January 17, 2020*
Published online: *May 11, 2020*

Key Words: *apoptosis; aquaporin-4; controlled cortical impact; edema; inflammation; protein S; TBI therapy; traumatic brain injury*

Chinese Library Classification No. *R453; R364; R741*

Introduction

Traumatic brain injury (TBI) is a vastly prevalent neurodegenerative disease affecting, in the United State alone, 1.5 million people annually (Algattas and Huang, 2013; Huang, 2013; Wang et al., 2017). Besides the initial physical affliction to the brain tissue, TBI triggers secondary pathologies including inflammatory response, perfusion disruption, local and peri-injury cell death, oxidative stress, and excitotoxicity that interact with and amplify each other to worsen the prognosis (Holmin et al., 1998; Holmin and Mathiesen, 2000; Loane and Faden, 2010; Shlosberg et al., 2010; Rodriguez-Rodriguez et al., 2014; Liang and Huang, 2017; Wofford et al., 2019; Sulhan et al., 2020). Brain edema, for example, causes increased intracranial pressure and reduced cerebral perfusion and oxygenation, which leads to further tissue degeneration and cell death (Unterberg et al., 2004; Shlosberg et al., 2010). In response to edema, brain tissue may self-correct imbalanced fluid homeostasis by upregulating aquaporin-4 (AQP4), the main water channel in the brain located in the astrocyte end-feet, to reabsorb vasogenic edema (Papadopoulos et al., 2004; Zhang et al., 2015). Another important player in secondary injury cascades is inflammatory response. TBI triggers the recruitment of $CD11b^+$ peripheral neutrophils and macrophages to the injury loci (Holmin and Mathiesen, 2000; Liu et al., 2018). Following infiltration, leukocytes release cytokines and increase oxidative stress, both of which activate microglia (Donat et al., 2017). Unlike the immediate and irreversible primary tissue damage, secondary injuries are delayed and reversible in nature, providing a therapeutic window to limit the progressive tissue damage. There is an unfulfilled and pressing need to develop a safe and effective neuroprotective treatment for acute-phase TBI, to mitigate the secondary damage that ensues (Stein and Wright, 2010). Given the complexity and diversity of TBI, multipotential drugs targeting various secondary injury mechanisms are in high demand (Loane and Faden, 2010).

As an important vitamin K-dependent blood coagulation regulator, protein S was first recognized as a nonenzymat-

ic cofactor for activated protein C (APC). APC is a serine protease that can downregulate coagulation by inactivating coagulation factors Va and VIIIa (Walker, 1980; Lundwall et al., 1986). Protein S increases APC-induced cleavage of Va by 20-fold and that of VIIIa by 3-fold (Rosing et al., 1995; O'Brien et al., 2000). Independent of APC, protein S also exerts inhibition of prothrombinase complex activity on endothelia and platelets by interacting directly with coagulation factors Va and Xa (Lundwall et al., 1986; Heeb et al., 1999). Besides its antithrombotic activity, protein S plays a role in inhibiting apoptosis, modulating inflammation, and stimulating phagocytosis through the activation of receptor tyrosine kinase TAM receptors (Tyro3, Axl, Mer) (Hafizi and Dahlback, 2006; van der Meer et al., 2014). The activation of TAM receptor further inhibits the Toll-like receptor mediated inflammatory response (Rothlin et al., 2007; Lemke and Rothlin, 2008).

In this study, we assessed the effects of intravenous administration of a single dose of human plasma protein S immediately following TBI by investigating the inflammatory response, edema formation, and behavioral outcomes in mouse controlled cortical impact (CCI) model of TBI.

Materials and Methods

CCI mouse model of TBI

Two to three-month-old male C57BL/6 mice (20–25 g) (Jackson Laboratories, Sacramento, CA, USA) were used in this study. Animal Use Protocols (AUPs) were approved by the University Committee on Animal Resources (UCAR) of University of Rochester Medical Center (approval No. UCAR-2008-102R) on November 12, 2013. AUPs were compliant with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All efforts have been made to reduce animal suffering.

Mice were randomly assigned to CCI-injured PBS-treated group, CCI-injured protein S-treated group, or sham-operated group. Brain injury was induced with a CCI device (Pittsburgh Precision Instruments, Pittsburgh, PA, USA) as previously described (Han et al., 2011; Tong et al., 2013). Briefly, anesthesia was induced with 2.5% isoflurane (Piramal Critical Care, Bethlehem, PA, USA) in a chamber, and maintained with 2% isoflurane using 33% O_2 and 65.5% N_2 delivery through a face mask. A heating pad (T/Pump professional, Stryker®, Kalamazoo, MI, USA) was placed underneath the mouse to keep a constant body temperature at 37.5°C. A right parietal craniotomy was performed. The dura mater was exposed. CCI was introduced perpendicularly to the brain in accordance with these specifications: diameter of impact tip = 3 mm; velocity of the impact = 6.7 m/s; duration of the impact = 100 ms; and displacement of the brain tissue = 1 mm. After introduction of CCI, Duragen® Dural Graft Matrix (Integra LifeSciences Corporation, Princeton, NJ, USA) was used in craniotomy, followed by immediate closure of the scalp. The CCI mouse model would then recover in a 37°C incubator until regaining of spontaneous motor activity. In the sham-operated group, CCI-induced injury was exempted after a right parietal craniotomy.

Protein S treatment

To evaluate the acute effect of protein S on TBI, we injected a single dose of 1 mg/kg protein S (human plasma-derived, Enzyme Research Laboratories, South Bend, IN, USA) into the tail vein of CCI-injured mice 10–15 minutes following the injury. In a different cohort of CCI-injured mice, sterile phosphate-buffered saline (PBS) of the volume that is identical to the volume of protein S injected was administrated through the tail vein as the vehicle control for the experiment.

Beam walk test

Beam walk test is an assay to evaluate fine motor coordination and balance (Yu et al., 2014). A 9 mm wide, 120 mm long narrow wooden beam was elevated 300 mm above the rubber foam. Mice were to walk across the narrow beam after being put on one end of the wooden beam. Contralateral to the impaired brain hemisphere are the left side front and hind limbs. The numbers of left side foot slippages were documented out of 30 steps (both front and hind limbs). The percentage of normal steps to all steps was calculated. For training purposes, mice were allowed to cross the beam twice prior to injury. Twenty-four hours after training, a baseline performance was assessed before injury. After surgery, the performance was evaluated on days 1, 2, and 3 following injury. Recovery ratio was calculated by normalizing the percentage of normal steps to the individual baseline. Final results from experimental groups were calculated by normalizing the recovery ratio of the CCI-injured group to that of the sham-operated group and presented as % of completion.

Rotarod test

The standard rotarod performance was evaluated to characterize motor coordination. Each mouse was trained on the rotarod device for 5 days prior to injury. The rotarod device (UGO-Basile, model 7650, Via Giuseppe Di Vittorio, Gemonio, Italy) consisted of a Plexiglas frame with a motorized rotating rod. The rod rotated at 5 r/min in the initial 60 seconds, then at 10 r/min from 60 to 160 seconds, and at 25 r/min from 160 to 240 seconds. Twenty-four hours prior to brain injury, rotarod tests were conducted to establish baseline latency for each mouse. The test phase consisted of two trials for each mouse. The average latency values were calculated based on the baseline evaluation and post-injury testing. The latencies in each group were averaged and presented in seconds and as a percentage of their respective baseline values from days 1 to 3 following injury.

Brain edema measurement

Brain water content was measured with the wet-dry method as previously described (McIntosh et al., 1990). After decapitation, the brains of mice were harvested and the cerebellums were discarded. Two hemispheres were separated, and each hemisphere was weighted and documented as wet weight. After complete desiccation in an oven at 108°C for 48 hours, each hemisphere was weighted and documented as dry weight. The percentage of water content of each hemi-

sphere (%H_2O) was calculated according to the following equation:

%H_2O = (wet weight – dry weight)/wet weight × 100

Tissue handling

Each mouse was deeply anesthetized with a ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail (intraperitoneal) and perfused transcardially with 50 mL PBS, followed by 50 mL of 4% paraformaldehyde for all histological analyses, including lesion volume analysis, immunohistochemistry staining, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Brain tissues were dissected, post-fixed overnight in 4% paraformaldehyde, cryoprotected in 15% and 30% sucrose solution, and subsequently embedded in Optimal Cutting Temperature (OCT) (Tissue-Tek®, Sakura Finetek USA, Torrance, CA, USA) for cryostat sectioning. Brain tissues were sectioned into 25 μm thick slices using cryostat, which were collected on slides and kept in a freezer at –80°C before use.

Lesion volume analysis

Twenty-four hours after TBI, six mice were randomly selected for lesion volume analysis from each group subjected to behavioral tests. Brain tissue was harvested at 0.5-mm intervals and then cut into 25-μm sections. Sections were mounted on slides and stained with 0.1% cresyl violet solution (cresyl violet acetate, Sigma, St. Louis, MO, USA). Morphometric image analysis was performed to determine the lesion volume (ImageJ software NIH, Bethesda, MD, USA). Lesion volume was measured first by drawing region of interests using the polygon tool and the measure tool (ImageJ), and then integrated to calculate the corresponding lesion volumes.

TUNEL staining

To detect the fragmented DNA of apoptotic cells, the Dead-EndTM Fluorometric TUNEL System (G3250, Promega Co, Madison, WI, USA) protocol was performed. Briefly, 25-μm sections were permeabilized with 0.2% Triton® X-100 in PBS for 10 minutes, rinsed three times with PBS, and then soaked in equilibration buffer for approximately 10 minutes at room temperature. These sections then underwent a terminal deoxynucleotidyl transferase (TdT) reaction for 1 hour at 37°C (90 μL equilibration buffer, 10 μL nucleotide mix, and 2 μL rTdT enzyme). Slides were merged in 2× SSC for 15 minutes to terminate the reaction, and then rinsed three times in PBS. Following the TUNEL staining procedure, the tissue was counterstained and coverslipped with Vectashield® Mounting Media (Vector Laboratories). Apoptotic neuronal nuclei were visualized in the brain tissue sections by stimulation with 520 nm light for fluorescein stains (green) and 460 nm light for DAPI stains (blue) using a fluorescence optical microscope (Olympus BX-51 with Olympus DP70 digital camera, Olympus, Tokyo, Japan) or a confocal microscope (Olympus IX81 FVF with Fluoview FV500, Olympus).

Immunohistochemistry

The brain tissue sections were thawed and air-dried for 30 minutes, and then washed with PBS (pH 7.4) for 5 minutes. After incubation in blocking solution (10% goat and 0.1% Triton® X-100) for 60 minutes, sections were incubated at 4°C overnight with one of the following primary antibodies: activated caspase-3 (1:300, rabbit polyclonal antibody, ab13847, Abcam, Cambridge, MA, USA), B-cell lymphoma 2 (Bcl-2) (C-2, 1:200, mouse monoclonal antibody, sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or CD11b (1:400, rat anti-mouse monoclonal antibody, IgG2b, AbD Serotec, Raleigh, NC, USA) diluted in 0.1% Triton® X-100 and 5% normal goat serum. On the following day, after being rinsed with PBS, brain sections were incubated at room temperature for 1 hour with the proper secondary antibodies: biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA), biotinylated horse anti-mouse antibody (1:200, Vector Laboratories), rabbit anti-rat antibody (1:200, Vector Laboratories) and goat anti-rabbit antibody (1:200, Vector Laboratories); then in 3% H_2O_2/PBS for 10 minutes. Avidin-biotin complex (ABC) (Vector Laboratories) was applied before cells were visualized with diaminobenzidine (DAB). After rinsing with distilled water for 5 minutes, sections were counterstained with Nissl stain, dehydrated in graded alcohol series and xylenes, and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). The cells were observed using Olympus BX-51 microscope with an Olympus DP70 digital camera. The numbers of positive cells for activated caspase-3, CD11b and Bcl-2 and positive areas for AQP4 in each brain section were quantified using the ImageJ analysis system, and the final result was presented as cell number per 0.5 mm^2.

Immunofluorescence

Staining for water channel AQP4 was carried out by similar procedures as in immunohistochemistry on the first day using the primary antibody targeting AQP4 (1:300, rabbit polyclonal antibody, AB3594, Millipore, Temecula, CA, USA). A secondary antibody for fluorescency (1:500, Cy3 donkey anti-rabbit, Jackson Immuno Research, West Grove, PA, USA) was used to detect the primary antibody on the following day. Vecta shield containing DAPI (Vector Laboratories) was used for mounting. Immunofluorescence was visualized with a Bio-Rad MRC500 confocal scanning microscope (Bio-Rad Laboratories, Hercules, CA, USA) attached to an inverted microscope (IX81, Olympus, Tokyo, Japan; operated with Olympus Fluoview 500) or a fluorescence optical microscope (Olympus BX-51 with Olympus DP70 digital camera). Cell counter function in imageJ was utilized to quantify cell number

Western blot assay

Brain tissues were rapidly removed after decapitation. The frontal segments from each separated hemisphere were kept frozen at –80°C for analysis. Tissue segments were homogenized after incubation in a buffer containing 1 mM (eth-

ylenediaminetetraacetic acid) EDTA, 1 mM dithiothreitol, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.9), 1 mM phenylmethylsulfonyl fluoride, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 10% glycerol, and 10% complete EDTA-free protease inhibitor (Roche Diagnostics) at 4°C for 30 minutes. Homogenates were centrifuged at 2348 × *g* for 10 minutes. Afterward, the supernatants were centrifuged at 13,523 × *g* for 30 minutes and frozen at –80°C, while the pellets were resuspended in the aforementioned buffer and centrifuged at 13,523 × *g* for 10 minutes. The Bradford method (Bio-Rad Laboratories) was used to determine protein concentrations in all extracts. Equal protein samples (70 μg) were separated on 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with 5% SDS stacking gels before transference onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Nonspecific antigen binding was blocked by incubation with 5% dry milk in PBS-Tween 20 (0.05%, Sigma) for 60 minutes. The membranes were washed three times with PBS-Tween 20 for 5 minutes each and subsequently incubated with rabbit polyclonal to active caspase-3 (1:500, Abcam, Cambridge, MA, USA) and β-actin primary antibodies (1:1000, Abcam), rabbit polyclonal to AQP4 (1:500, Millipore, Hayward, CA, USA) overnight at 4°C. Peroxidase-coupled goat anti-rabbit IgG (1:5000, Abcam) was used for secondary incubations for 1 hour at room temperature. Routine enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize reactive bands. Immunoreactive bands indicating expression of active caspase-3 (17 kDa) and AQP4 (38 kDa) were detected in mouse brain lysates. The optical density (OD) of reactive bands was quantified by image J (NIH, Bethesda, MD, USA). Protein levels were depicted as the OD of the targeted factor relative to β-actin within the same lane.

Real-time PCR

After mice were sacrificed, dissected parietal cortical tissues were frozen on dry ice. TRIzol® reagent (Invitrogen, Waltham, MA, USA) was used to prepare total RNA. The RNA product was redissolved in 100 μL of RNase-free water and then cleaned by the RNeasy Mini Kit (74104, Qiagen, Valencia, CA, USA). Synthetization of cDNA was performed with High Capacity cDNA Reverse Transcription Kit (with RNase inhibitor, Applied BioSystems, Waltham, MA, USA) following standard protocol. For control, MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific) was substituted with nuclease-free water. The expression level of each target gene was quantified as relative to GADPH using the Applied Biosystems 7000 Real-Time PCR System (Applied BioSystems, Inc, Waltham, MA, USA). The following primer pairs were utilized for target mRNA amplification: AQP4: Mm00802131_ml, interleukin-1 (IL-1β): Mm01336189_ml, tumor necrosis factor (TNF-α): Mm00443258_ml, caspase-3: Mm01195084_ml, and mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an endogenous control: 4352932E (Applied Biosystems, Inc, Foster City, CA, USA). The comparative threshold (ΔCt) method was used, and data were analyzed with ABI PRISM® 7000 SDS Software SDS V 1.2 (Applied Biosystems, Inc.)

Statistical analysis

Statistical analysis was conducted with GraphPad Prism5 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare data among groups. Two tailed unpaired *t*-test was used to compare data between two groups. A *P*-value less than 0.05 was accepted as statistically significant. All data collected for function evaluations and cell analyses are presented as the mean ± standard deviation (SD).

Results

Single protein S administration partially prevents motor function aggravation in the acute phase following TBI

All CCI-injured animals displayed severe motor function deficits, especially in the left hind limbs. Motor function difference between the protein S-treated and the PBS-treated groups was the most prominent on post-injury day 1. The protein S-treated group showed a significant 56% increase in left front limb performance in the beam walk test compared to that in the PBS-treated group (protein S-treated group: 46.42 ± 19.82% *vs.* PBS-treated group: 25 ± 13.47%, $P < 0.01$) (**Figure 1**). By post-injury day 3, the performance in the protein S-treated and PBS-treated groups recovered to approximately 50% of that in the sham-operated group (**Figure 1**), while the left hind limbs remained paralyzed in both injured groups. Rotarod testing of latency did not differ significantly between these groups within the tested period, even on post-injury day 1 (24 hours post-injury: protein S-treated group: 51.48 ± 31.84% *vs.* PBS-treated group: 45.37 ± 32.7%, $n = 12$, $P > 0.05$).

Protein S treatment decreases edema and prevents against progressive tissue damage

At 24 hours post-injury, both CCI-injured groups demonstrated significantly higher brain water content in the ipsilesional hemisphere compared to the sham-operated group (sham-operated group: 78.44 ± 0.12% *vs.* protein S-treated group: 79.46 ± 0.15%, $P < 0.001$; PBS-treated: 80.64 ± 0.23%, $P < 0.001$) (**Figure 2A**). However, CCI-injured mice treated with protein S had significantly less severe edema compared to the mice treated with PBS ($P < 0.001$). No statistical difference in the contralateral hemispheres was observed among CCI-injured mice with or without protein S and sham-operated control ($P > 0$ .05).

To evaluate the effects of protein S treatment on progressive tissue loss, we measured lesion volume 24 hours after injury. Injured mice treated with protein S showed a substantial 33% decrease in lesion volume compared to their PBS-treated counterparts (protein S-treated group: 3.82 ± 0.67 mm^3 *vs.* PBS-treated: 5.72 ± 0.53 mm^3, $P < 0.01$; **Figure 2B**).

Inflammation and apoptosis are reduced by acute protein S treatment following TBI

To determine whether human protein S could inhibit inflam-

mation in TBI, we assayed for leukocyte infiltration into the injured brain. Immunohistochemistry staining showed that CD11b+ leukocyte infiltration decreased dramatically in and around the injured brain cortex in protein S-treated animals compared to those administered with PBS (protein S-treated group: 166 ± 53.24 *vs*. PBS-treated group: 606 ± 87.43, $P < 0.001$; **Figure 3**). We used the TUNEL assay to test whether acute protein S treatment following TBI mitigates apoptosis. TUNEL staining revealed that protein S treatment reduced the number of apoptotic cells in the mouse brains compared to PBS treatment (protein S-treated group: 124 ± 26.08 *vs*. PBS-treated group: 238 ± 47.84, $P < 0.01$; **Figure 4**).

We asked whether decreased post-injury apoptosis in protein S-treated animals was achieved through the inhibition of activated caspase-3 (a critical "executioner" of apoptosis). This hypothesis was tested by comparing both the number of cells that are positive for activated caspase-3 and the protein level of activated caspase-3 in injured animals treated with either protein S or PBS. Protein S treatment significantly lowered the number of activated caspase-3 positive cells in the injured cortex (protein S-treated group: 294 ± 61.86 *vs*. PBS-treated group: 563 ± 76.93, $P < 0.001$) (**Figure 5A–C**). Western blot assay results confirmed the presence of activated caspase-3 in the ipsilesional hemisphere with significantly higher levels in mice not treated with protein S (protein S-treated group: 0.74 ± 0.09 *vs*. PBS-treated group: 1.134 ± 0.30, $P < 0.05$) (**Figure 5D** and **E**).

To further demonstrate the role of protein S in inhibiting apoptosis after TBI, we examined the level of anti-apoptotic protein Bcl-2 by counting the number of Bcl-2-positive cells in both treated and untreated groups. We found that the amount of cells expressing Bcl-2 increased prominently in protein S-treated group compared to the PBS-treated group (protein S-treated group: 532 ± 102.58 *vs*. PBS-treated group: 313 ± 66.81, $P < 0.01$) (**Figure 6**).

To further illuminate the mechanisms that contributed to the beneficial effects of protein S on inflammation and apoptosis after TBI, mRNA levels of pro-inflammatory cytokines IL-1 β and TNF-α were measured with quantitative PCR (qPCR) in brain tissue samples from all three groups. Compared to sham-operated animals, the expression levels of these inflammatory mediators increased approximately 50-fold by 24 hours after TBI (TNF-α: protein S-treated group: 51.12 ± 16.83 fold *vs*. PBS-treated group: 55.45 ± 22.78, $n = 5$, $P > 0.05$; IL-1β: protein S-treated group: 50.54 ± 30.58 fold *vs*. PBS-treated group: 39.84 ± 22.41 fold, $n = 5$, $P > 0.05$) (data not shown). However, protein S treatment did not alter the mRNA levels of these inflammatory mediators after TBI.

Protein S increases AQP-4 expression after TBI

Based on the finding that protein S treatment decreases brain edema after TBI, we asked whether protein S affected AQP4 expression. Indeed, in the ipsilesional cortex, qPCR results showed significantly higher AQP4 mRNA level in the protein S-treated group compared to the PBS-treated group (protein S-treated group: 2.84 ± 0.45 *vs*. PBS-treated group: 2.08 ± 0.39, $P < 0.05$; **Figure 7E**). On the contralateral side, no significant difference was found between these groups (1.19 ± 0.07 *vs*. 1.38 ± 0.33, $P > 0.05$). This finding was confirmed by immunofluorescent analysis of AQP4 in and around the injury loci (protein S-treated group: 23.6 ± 1.45% *vs*. PBS-treated group: 15.03 ± 0.73%, $P < 0.001$) (**Figure 7A–D**), and by western blot assay of AQP4 expression in the ipsilateral hemispheres ($P < 0.01$) (**Figure 7F** and **G**) in post-injury mice treated with either protein S or PBS.

Discussion

The present study showed that protein S treatment immediately (10–15 minutes) following TBI reduced the number of $CD11b^+$ cells in the injured hemisphere. Protein S treatment also reduced cell death by decreasing caspase-3 activity while increasing Bcl-2 expression. We also found that protein S treatment reduced brain edema probably through the upregulation of AQP4. As a result of diminished secondary injuries, protein S treated mice had decreased progressive tissue loss and performed better in fine motor tests compared to the untreated counterparts. Our data demonstrate that a single dose of intravenous protein S treatment immediately after CCI prevented, to a certain extent, the worsening effect of TBI caused by secondary injuries and maintained a better neurological function in the early stage.

In the beam walk test, the protein S-treated group performed significantly better than the vehicle-treated group. This advantage was maintained for the experimental duration. The fine motor function of the PBS-treated group slowly and gradually recovered over time, indicating that intrinsic post-injury neuro-regenerative events were taking place. That protein S did not further improve the fine motor function in the treated animals is also suggestive of its role in reducing the secondary effects rather than aiding in regeneration. Moreover, given the same parameters, CCI creates highly reproducible primary injuries and the tissue losses after injury are also consistent (Han et al., 2011). The fact that the PBS-treated group had significantly higher tissue loss at 1 day post-injury compared to the protein S-treated group suggests that more severe secondary tissue loss occurred in the PBS-treated group.

As a multi-functional factor, protein S has the potential to restore perfusion as well as diminishing apoptosis and inflammatory response after central nervous system injury. Indeed, protein S was found to be beneficial in ischemic injury caused by stroke. Liu et al. (2003) showed intravenous delivery of human protein S immediately after the initiation of middle cerebral artery occlusion followed by reperfusion significantly improved neuronal survival and motor function recovery while reduced infarction, brain fibrin deposition and neutrophil infiltration. Zhu et al. (2010) demonstrated that protein S treatment has a beneficial impact on maintaining blood-brain barrier integrity in the setting of hypoxic/ischemic insults. Moreover, protein S might exert a favorable effect by increasing APC activity. APC has been found to be neuroprotective in murine ischemic stroke model and in hypoxic human brain endothelium (Cheng et al., 2003; Fernandez et al., 2003).

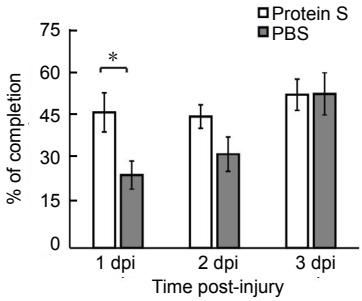

Figure 1 Acute phase post-injury motor function in mice after traumatic brain injury is improved by protein S administration.
Bar graph shows the motor function of left front limb after controlled cortical impact evaluated by beam walk test. At 1 day post-injury (dpi), the protein S-treated group showed significantly better left front limb movement compared to the PBS-treated group (n = 12, P < 0 .05). The motor function of the PBS-treated group recovered slowly over time while the motor function of the protein S-treated group was consistently better. ∗P < 0.05 (one-way analysis of variance followed by two tailed unpaired t-test).

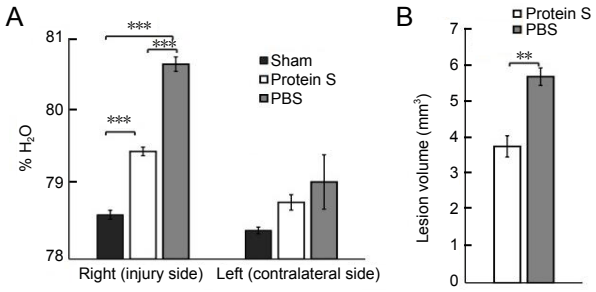

Figure 2 Brain edema and progressive tissue damage in the brain of traumatic brain injury mice are reduced by protein S treatment.
(A) The percentage of brain water content in both hemispheres on post-injury day 1. In the ipsilesional hemisphere, both injured groups had significantly higher water content than the sham-operated group (n = 5, P < 0.001). With protein S treatment, mice had significantly decreased edema compared to PBS treatment (n = 5, P < 0.001). No statistical difference was observed in the contralateral hemisphere. (B) Lesion volume on post-injury day 1 from both protein S-treated and -untreated groups. Protein S-treated group showed significantly smaller lesion volume compared to the PBS-treated group (n = 5, P < 0.01). ∗∗P < 0.01, ∗∗∗P < 0.001. One-way analysis of variance followed by two tailed unpaired t-test was used.

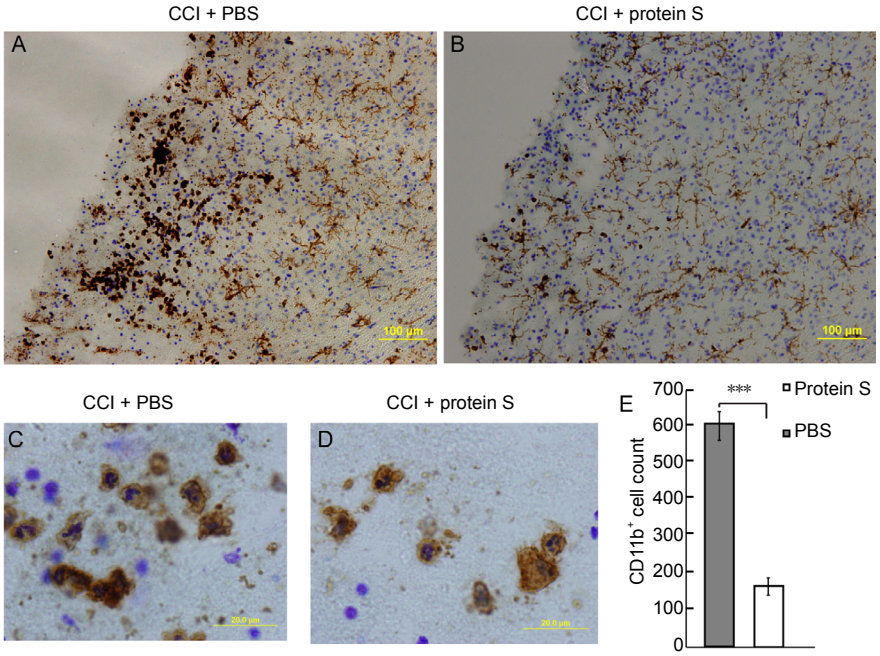

Figure 3 Protein S treatment decreases inflammatory response 24 hours in the brain of mice after traumatic brain injury (TBI).
(A, B) Immunohistochemistry of CD11b$^+$ leukocyte infiltration in and around the injured brain cortex of animals treated with protein S (B) compared to animals treated with PBS (A) (scale bars: 100 μm). (C, D) Higher magnification images from PBS-treated group (C) and protein S-treated group (D) (scale bars: 20 μm). (E) Counts of CD11b$^+$ cells from protein S- and PBS-treated groups 24 hours after injury. Protein S treatment significantly reduced CD11b$^+$ cell numbers (n = 5, P < 0.001). ∗∗∗P < 0.001. Two tailed unpaired t-test was used. CCI: Controlled cortical impact.

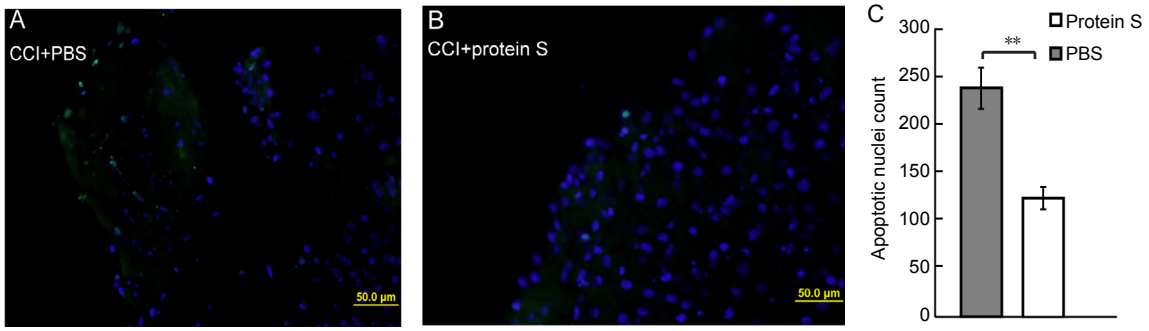

Figure 4 Protein S treatment reduces the number of apoptotic cells in the brain of mice after TBI.
(A, B) Representative images of TUNEL staining (green) and counterstaining with DAPI (blue) from PBS-treated (A) or protein S-treated groups (B) (scale bars: 50 μm). (C) TUNEL-positive nuclei counts from both protein S-treated and PBS-treated groups. Results showed less positive cells in the brains of the protein S-treated group compared to the PBS-treated group (n = 5, P < 0.01). ∗∗P < 0.01. Two tailed unpaired t-test was used. CCI: Controlled cortical impact.

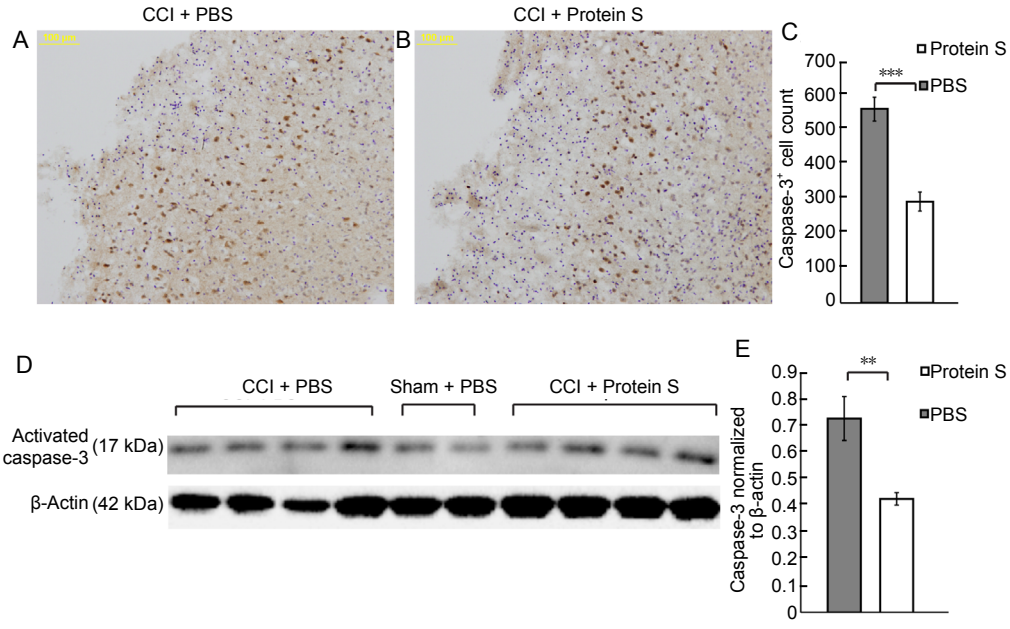

Figure 5 Protein S decreases caspase-3 levels in the injured cortex of mice after traumatic brain injury.
After protein S administration, activated caspase-3 expression in injured cortex was measured by both immunohistochemistry (A–C) and western blot assay (D, E). (A, B) Representative images of injured cortex treated with PBS (A) and protein S (B). Scale bars: 100 μm. (C) Quantification of cell numbers from both the protein S-treated group and the PBS-treated group, showing significantly decreased caspase-3-positive cell number in the protein S-treated group (n = 5, P < 0.001). (D) Representative gel image from western blot assay. (E) Quantification of western blot showing significantly decreased active caspase-3 protein expression in the protein S-treated group (n = 5, P < 0.01). **P < 0.01, ***P < 0.001. Two tailed unpaired t-test was used. CCI: Controlled cortical impact.

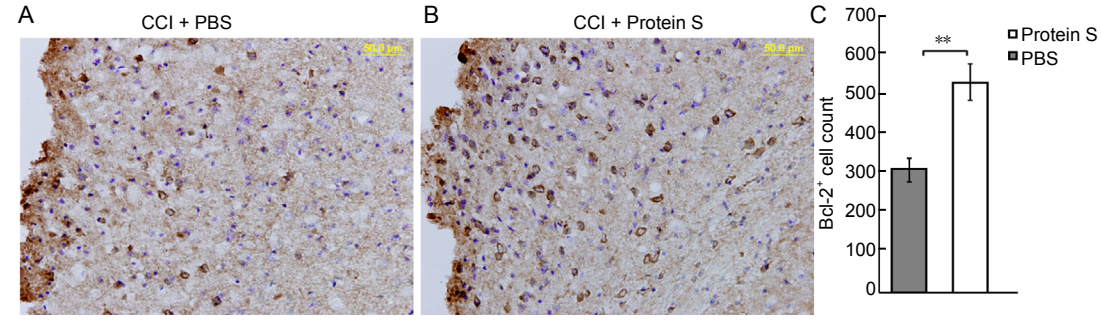

Figure 6 Protein S administration increases levels of Bcl-2 in injured cortex of mice after traumatic brain injury.
(A) Representative immunohistochemical staining of Bcl-2 counter stained with Nissl staining in PBS- (A) and protein S- (B) treated injured cortex. Scale bars: 100 μm. (C) Quantification of Bcl-2-positive cell number in both protein S- and PBS-treated groups. Bcl-2-positive cell number in protein S-treated group was significantly higher compared to the PBS-treated group (n =5, P < 0.01). **P < 0.01. Two tailed unpaired t-test was used. CCI: Controlled cortical impact.

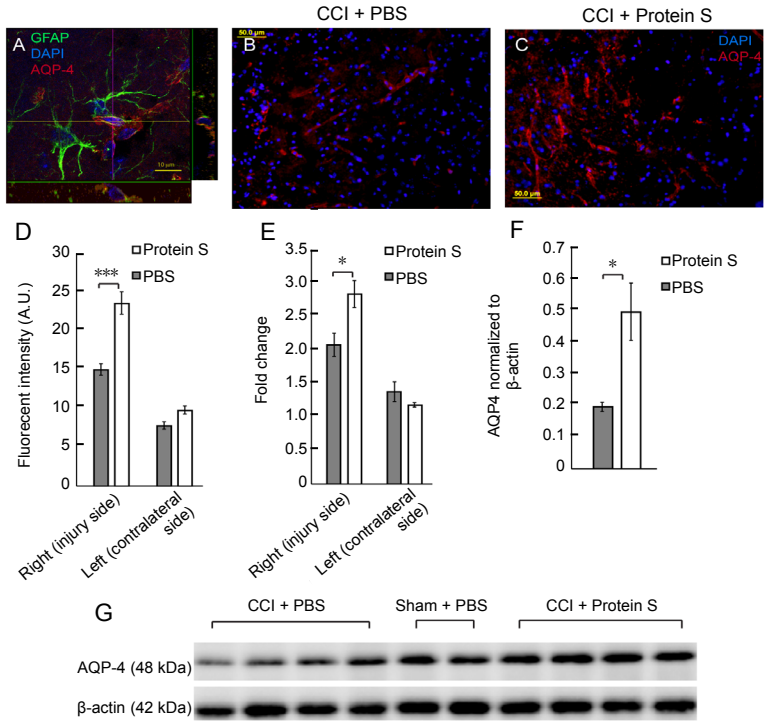

Figure 7 Protein S treatment increases aquaporin-4 (AQP4) expression in injured cortex of mice after traumatic brain injury (TBI).
(A) Confocal microscope images showing aquaporin-4 (red) expression in astrocytes (green) end-feet, which surround capillaries in the brain and help to eliminate water from the extracellular space after brain injury (scale bar: 10 μm). (B, C) Representative AQP4 staining in the cortex from PBS-treated (B) and protein S-treated groups (C) (scale bar: 50 μm). (D) Quantification of AQP4 positive staining in both ipsilateral and contralateral hemispheres from animals treated either with protein S or PBS. Protein S treatment 24 hours after TBI significantly increases AQP4 positive staining compared to the PBS treatment group in the injured hemisphere (ipsilateral) (n = 5, P < 0.001). (E) Real time-PCR showing that 24 hours after traumatic brain injury, AQP4 mRNA level increased 2–3 fold after protein S treatment compared to PBS treatment especially in the ipsilateral hemispheres (n = 5, P < 0.05). (F) At the protein level, western blot quantification showed the same observation that AQP4 increased after protein S treatment (n = 5, P < 0.05). (G) Representative western blot gel image. *P < 0.05, ***P < 0.001. Two tailed unpaired t-test was used. CCI: Controlled cortical impact.

Our results revealed that protein S reduced the inflammatory response and apoptosis in the brain trauma setting. Significantly less leukocyte infiltration was observed in the ipsilesional cortex of the protein S-treated group. These CD11b+ peripheral leukocytes were mostly neutrophils judged by their morphology but also included macrophages. Local microglials in the cortex of the vehicle-treated group seemed to display activated morphology. Further examination with Sholl analysis or other morphology characterizations could be done to quantify this general observation (Heindl et al., 2018). The amelioration of edema also supports the anti-inflammatory effect of protein S. Besides the invasion of circulating immune cells, another key feature of central nervous system inflammation is the activation of pro-inflammatory cytokines and chemokines (Lucas et al., 2006; Chitnis and Weiner, 2017). Elevation of IL-1β and TNF-α have been consistently reported in different TBI models (Chitnis and Weiner, 2017). In line with these findings, we found almost 50-fold increase of IL-1β and TNF-α in mRNA levels in our mouse CCI model. A single acute administration of human protein S, however, did not impede the upsurge of neither IL-1β nor TNF-α. Earlier *in vitro* findings suggested that TAM receptors suppress innate immune response by inhibiting Toll-like receptors; and that $Tyro3^{-/-}$, $Axl^{-/-}$ and $Mer^{-/-}$ cell lines exhibited a two- to four-fold increase of the TNF-α level when Toll-like receptors were activated by CpG or lipopolysaccharide (Rothlin et al., 2007). Since protein S is a TAM receptor activator, we expected that the level of pro-inflammatory cytokines would decrease after protein S treatment. Interestingly, no difference has been observed in cytokine levels between the protein S-treated and untreated groups (TNF-α and IL-1β). This contradiction could be attributed to many reasons, one of which is the intrinsic difference between the multifold TBI stressed *in vivo* model and the clean pharmacological stressed *in vitro* model. Moreover, we examined the mRNA level with qPCR while the earlier group tested the protein level with enzyme-linked immunosorbent assay (ELISA). The observation that a single dose of protein S treatment following TBI did not prevent the upsurge of IL-1β or TNF-α also raised the question whether multiple treatments could be further beneficial.

Besides functioning as an immune-suppressor, protein S decreased cell death when given immediately after injury. Similar to that found by Zhu et al. (2010) in an excitotoxicity model in cortical neurons, we demonstrated that protein S decreased the level of apoptosis executioner protein exactivated caspase-3. Moreover, protein S treatment leads to a higher level of anti-apoptotic protein Bcl-2.

Our results also suggests that protein S treatment reduced brain edema, the time course of which coincides with the upregulation of AQP4. The endogenous restorative response to brain injury includes the substantially increased expression of AQP4 in the peri-injury region following the insult (Neal et al., 2007; Li et al., 2013). In the setting of a disrupted blood-brain barrier, as is in the case with brain trauma, AQP4 has been demonstrated to have beneficial effects by allowing the removal of excess water (Tait et al., 2010; Plog et al., 2015; Mestre et al., 2018). This has been demonstrated in AQP4 knockout mice, which, due to the loss of a water removal mechanism, have increased brain water content, elevated intracranial pressure, and scored lower on neurological assessment compared to wild-type mice with subarachnoid hemorrhage (Tait et al., 2010). The AQP4 facilitates the recovery of fluid homeostasis by redistributing excess extracellular water to the capillaries and peripheral circulation. This has warranted the upregulation of AQP4 expression as a novel therapeutic intervention in the case of brain edema (Papadopoulos et al., 2004; Tait et al., 2010). To our knowledge, this is the first study to provide evidence that protein S promotes post-injury expression of AQP4, which in turn reduces TBI-related edema. The molecular pathway underlying such an effect, however, requires further investigation. Recent studies suggested that the subcellular location of AQP4 is crucial for the resolution of edema after TBI (Iliff et al., 2012, 2014; Ren et al., 2013), and the posttraumatic upregulation of AQP4 does not necessarily localize to the end-feet of astrocyte (Ren et al., 2013). It would also be intriguing to evaluate whether protein S restores the subcellular localization of AQP4.

Conclusion

Protein S is a natural anticoagulant with multiple functions that could be beneficial for the prognosis of TBI. Our data demonstrate that intravenous protein S treatment immediately following TBI suppresses three major secondary injury cascades being apoptotic cell death, inflammation, and edema formation. Therefore, protein S may hold a great therapeutic potential for TBI patients.

Author contributions: *XW, JT, XH, and JHH designed the study, searched literature, and prepared the initial manuscript. XW, JT, XH, XQ and JZ carried out experiments and collected data. JT and XH carried out statistical analysis. EW, XQ, and JHH edited the manuscript. All authors commented on previous versions of the manuscript and approved the final manuscript.*
Conflicts of interest: *The authors report no conflict of interest regarding the materials, methods or findings specified in this study.*
Financial support: *This work was supported in part by a University of Rochester Institutional Grant (2011NSG-Huang; to JHH), National Institute of Health (NIH-R01-NS-067435; to JHH), and the Hellen Vosberg McCrillus Plummer and Robert Edward Lee Plummer, Jr, Endowment fund from Baylor Scott & White Medical Center (to JHH).*
Institutional review board statement: *All Animal Use Protocols were approved by the University Committee on Animal Resources of the University of Rochester Medical Center (approval No. UCAR-2008-102R) on November 12, 2013.*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewers: *Andrew Octavian Sasmita, Georg-August-University Göttingen, Germany; Dario Siniscalco, Università degli studi della*

Campania "Luigi Vanvitelli", Italy.
Additional file: *Open peer review reports 1 and 2.*

References

Algattas H, Huang JH (2013) Traumatic Brain Injury pathophysiology and treatments: early, intermediate, and late phases post-injury. Int J Mol Sci 15:309-341.

Cheng T, Liu D, Griffin JH, Fernandez JA, Castellino F, Rosen ED, Fukudome K, Zlokovic BV (2003) Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. Nat Med 9:338-342.

Chitnis T, Weiner HL (2017) CNS inflammation and neurodegeneration. J Clin Invest 127:3577-3587.

Donat CK, Scott G, Gentleman SM, Sastre M (2017) Microglial activation in traumatic brain injury. Front Aging Neurosci 9:208.

Fernandez JA, Xu X, Liu D, Zlokovic BV, Griffin JH (2003) Recombinant murine-activated protein C is neuroprotective in a murine ischemic stroke model. Blood Cells Mol Dis 30:271-276.

Hafizi S, Dahlback B (2006) Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J 273:5231-5244.

Han X, Tong J, Zhang J, Farahvar A, Wang E, Yang J, Samadani U, Smith DH, Huang JH (2011) Imipramine treatment improves cognitive outcome associated with enhanced hippocampal neurogenesis after traumatic brain injury in mice. J Neurotrauma 28:995-1007.

Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH (1999) C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem 274:36187-36192.

Heindl S, Gesierich B, Benakis C, Llovera G, Duering M, Liesz A (2018) Automated morphological analysis of microglia after stroke. Front Cell Neurosci 12:106.

Holmin S, Mathiesen T (2000) Intracerebral administration of interleukin-1beta and induction of inflammation, apoptosis, and vasogenic edema. J Neurosurg 92:108-120.

Holmin S, Soderlund J, Biberfeld P, Mathiesen T (1998) Intracerebral inflammation after human brain contusion. Neurosurgery 42:291-299.

Huang JH (2013) Traumatic brain injury. Neurol Res 35:221-222.

Iliff JJ, Chen MJ, Plog BA, Zeppenfeld DM, Soltero M, Yang L, Singh I, Deane R, Nedergaard M (2014) Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. J Neurosci 34:16180-16193.

Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA, Nagelhus EA, Nedergaard M (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. Sci Transl Med 4:147ra111.

Lemke G, Rothlin CV (2008) Immunobiology of the TAM receptors. Nat Rev Immunol 8:327-336.

Li X, Han Y, Xu H, Sun Z, Zhou Z, Long X, Yang Y, Zou L (2013) Aquaporin 4 expression and ultrastructure of the blood-brain barrier following cerebral contusion injury. Neural Regen Res 8:338-345.

Liang B, Huang JH (2017) Elevated NKCC1 transporter expression facilitates early post-traumatic brain injury seizures. Neural Regen Res 12:401-402.

Liu D, Guo H, Griffin JH, Fernandez JA, Zlokovic BV (2003) Protein S confers neuronal protection during ischemic/hypoxic injury in mice. Circulation 107:1791-1796.

Liu YW, Li S, Dai SS (2018) Neutrophils in traumatic brain injury (TBI): friend or foe? J Neuroinflammation 15:146.

Loane DJ, Faden AI (2010) Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. Trends Pharmacol Sci 31:596-604.

Lucas SM, Rothwell NJ, Gibson RM (2006) The role of inflammation in CNS injury and disease. Br J Pharmacol 147 Suppl 1:S232-240.

Lundwall A, Dackowski W, Cohen E, Shaffer M, Mahr A, Dahlback B, Stenflo J, Wydro R (1986) Isolation and sequence of the cDNA for human protein S, a regulator of blood coagulation. Proc Natl Acad Sci U S A 83:6716-6720.

McIntosh TK, Soares H, Thomas M, Cloherty K (1990) Development of regional cerebral oedema after lateral fluid-percussion brain injury in the rat. Acta Neurochir Suppl (Wien) 51:263-264.

Mestre H, Hablitz LM, Xavier AL, Feng W, Zou W, Pu T, Monai H, Murlidharan G, Castellanos Rivera RM, Simon MJ, Pike MM, Plá V, Du T, Kress BT, Wang X, Plog BA, Thrane AS, Lundgaard I, Abe Y, Yasui M, et al. (2018) Aquaporin-4-dependent glymphatic solute transport in the rodent brain. Elife 7.

Neal CJ, Lee EY, Gyorgy A, Ecklund JM, Agoston DV, Ling GS (2007) Effect of penetrating brain injury on aquaporin-4 expression using a rat model. J Neurotrauma 24:1609-1617.

O'Brien LM, Mastri M, Fay PJ (2000) Regulation of factor VIIIa by human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. Blood 95:1714-1720.

Papadopoulos MC, Manley GT, Krishna S, Verkman AS (2004) Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. FASEB J 18:1291-1293.

Plog BA, Dashnaw ML, Hitomi E, Peng W, Liao Y, Lou N, Deane R, Nedergaard M (2015) Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. J Neurosci 35:518-526.

Ren Z, Iliff JJ, Yang L, Yang J, Chen X, Chen MJ, Giese RN, Wang B, Shi X, Nedergaard M (2013) 'Hit & Run' model of closed-skull traumatic brain injury (TBI) reveals complex patterns of post-traumatic AQP4 dysregulation. J Cereb Blood Flow Metab 33:834-845.

Rodriguez-Rodriguez A, Egea-Guerrero JJ, Murillo-Cabezas F, Carrillo-Vico A (2014) Oxidative stress in traumatic brain injury. Curr Med Chem 21:1201-1211.

Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Varadi K, Schwarz HP, Tans G (1995) Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem 270:27852-27858.

Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G (2007) TAM receptors are pleiotropic inhibitors of the innate immune response. Cell 131:1124-1136.

Shlosberg D, Benifla M, Kaufer D, Friedman A (2010) Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol 6:393-403.

Stein DG, Wright DW (2010) Progesterone in the clinical treatment of acute traumatic brain injury. Expert Opin Investig Drugs 19:847-857.

Sulhan S, Lyon KA, Shapiro LA, Huang JH (2020) Neuroinflammation and blood-brain barrier disruption following traumatic brain injury: Pathophysiology and potential therapeutic targets. J Neurosci Res 98:19-28.

Tait MJ, Saadoun S, Bell BA, Verkman AS, Papadopoulos MC (2010) Increased brain edema in aqp4-null mice in an experimental model of subarachnoid hemorrhage. Neuroscience 167:60-67.

Tong J, Liu W, Wang X, Han X, Hyrien O, Samadani U, Smith DH, Huang JH (2013) Inhibition of Nogo-66 receptor 1 enhances recovery of cognitive function after traumatic brain injury in mice. J Neurotrauma 30:247-258.

Unterberg AW, Stover J, Kress B, Kiening KL (2004) Edema and brain trauma. Neuroscience 129:1021-1029.

van der Meer JH, van der Poll T, van 't Veer C (2014) TAM receptors, Gas6, and protein S: roles in inflammation and hemostasis. Blood 123:2460-2469.

Walker FJ (1980) Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem 255:5521-5524.

Wang F, Wang X, Shapiro LA, Cotrina ML, Liu W, Wang EW, Gu S, Wang W, He X, Nedergaard M, Huang JH (2017) NKCC1 up-regulation contributes to early post-traumatic seizures and increased post-traumatic seizure susceptibility. Brain Struct Funct 222:1543-1556.

Wofford KL, Loane DJ, Cullen DK (2019) Acute drivers of neuroinflammation in traumatic brain injury. Neural Regen Res 14:1481-1489.

Yu K, Wu Y, Zhang Q, Xie H, Liu G, Guo Z, Li F, Jia J, Kuang S, Hu R (2014) Enriched environment induces angiogenesis and improves neural function outcomes in rat stroke model. J Neurol Sci 347:275-280.

Zhang C, Chen J, Lu H (2015) Expression of aquaporin-4 and pathological characteristics of brain injury in a rat model of traumatic brain injury. Mol Med Rep 12:7351-7357.

Zhu D, Wang Y, Singh I, Bell RD, Deane R, Zhong Z, Sagare A, Winkler EA, Zlokovic BV (2010) Protein S controls hypoxic/ischemic blood-brain barrier disruption through the TAM receptor Tyro3 and sphingosine 1-phosphate receptor. Blood 115:4963-4972.

P-Reviewers: Sasmita AO, Siniscalco D; C-Editor: Zhao M; S-Editor: Li CH; L-Editor: Song LP; T-Editor: Jia Y