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Effect of slosh mitigation on histologic markers of traumatic brain injury

Laboratory investigation

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Object. Helmets successfully prevent most cranial fractures and skull traumas, but traumatic brain injury (TBI) and concussions continue to occur with frightening frequency despite the widespread use of helmets on the athletic field and battlefield. Protection against such injury is needed. The object of this study was to determine if slosh mitigation reduces neural degeneration, gliosis, and neuroinflammation.

Methods. Two groups of 10 adult male Sprague-Dawley rats were subjected to impact-acceleration TBI. One group of animals was fitted with a collar inducing internal jugular vein (IJV) compression prior to injury, whereas the second group received no such collar prior to injury. All rats were killed 7 days postinjury, and the brains were fixed and embedded in paraffin. Tissue sections were processed and stained for markers of neural degeneration (Fluoro-Jade B), gliosis (glial fibrillary acidic protein), and neuroinflammation (ionized calcium binding adapter molecule 1).

Results. Compared with the controls, animals that had undergone IJV compression had a 48.7%–59.1% reduction in degenerative neurons, a 36.8%–45.7% decrease in reactive astrocytes, and a 44.1%–65.3% reduction in microglial activation.

Conclusions. The authors concluded that IJV compression, a form of slosh mitigation, markedly reduces markers of neurological injury in a common model of TBI. Based on findings in this and other studies, slosh mitigation may have potential for preventing TBI in the clinical population.
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Key Words • **traumatic brain injury** • **slosh** • **rat** • **jugular vein compression**

Traumatic brain injury remains an immense public health problem and a leading cause of morbidity and death in persons younger than 45 years old in the US and industrialized countries.[6] Treatment options for TBI remain relatively limited in scope, emphasizing control of edema and ICP and subsequent rehabilitation. While numerous therapeutic agents such as progesterone[4,21,22,25] and docosahexaenoic acid[3,14,15] have been found to be neuroprotective in preclinical models, and some are currently the focus of clinical trials, few alternatives exist for the clinical management of TBI. Furthermore, it is unlikely that any treatment aimed at injury reversal will be as effective as injury prevention.

Efforts aimed at preventing TBI have continued to focus on extracranial protection in the form of helmets and improved helmet technology such as innovative shapes,

Abbreviations used in this paper: DPBS = Dulbecco phosphate-buffered saline; FJB = Fluoro-Jade B; GFAP = glial fibrillary acidic protein; Iba-1 = ionized calcium binding adapter molecule 1; ICP = intracranial pressure; IJV = internal jugular vein; TBI = traumatic brain injury.

shell materials, and padding. The emphasis on helmet technology may not be ideal given the limitations of helmets in preventing acceleration-deceleration forces causing axonal injury and subsequent concussion. Numerous species in nature, such as the woodpecker, seem to possess protective mechanisms that prevent the occurrence of repetitive TBI despite the inherent impacts associated with naturally exhibited behaviors. The ability of these species to prevent repetitive TBI may provide insight into the development of technologies for TBI prevention in humans.

The woodpecker anatomy is notable for a multitude of reasons ranging from a small subdural space to a unique tongue structure.[13,28,29] The tongue is notable in that it extends posteriorly from the base of the mouth, around the neck, over the occiput, and into the right nostril.[13] This arrangement, in which a muscular sling is formed in the neck, has been identified as a possible shock absorber by limiting motion in the neck.[13] In fact, the long tongue and hyoid bone are subject to significant stress during collision, as is evident in biomechanical studies.[28] Wang et al.[28] have described the hyoid bone as playing the role of a safety belt for the head of the woodpecker.

While human anatomy clearly differs from that of the woodpecker, some correlates do exist in the prevention of TBI. For example, professional boxers are capable of sustaining forces of great magnitude when prepared for the impact but suffer deleterious consequences when the blow is unexpected. Much of this protection has been attributed to a tightening of the neck muscles, one of which is the omohyoid muscle. The omohyoid muscle is unique in that it consists of 2 muscle bellies joined by a tendon and closely overlies and crosses the IJV, a rare anatomical relationship. Notably, contraction of the omohyoid muscle on yawning results in IJV compression and subsequent changes to intracerebral venous hemodynamics.[16] Therefore, contraction of the omohyoid muscle, as well as other muscles in the neck, may have a protective effect in humans through stabilization of the neck, much like the tongue and hyoid bone in the woodpecker. Furthermore, contraction of the omohyoid muscle alters intracerebral hemodynamics, which may serve as a protective mechanism by increasing intracranial volume and reducing intracranial movement, minimizing collisions incurred by the brain within the skull.

In an earlier study[24] we postulated that a reduction in intracranial compliance via increased intracerebral volume would result in reduced brain injury. This theory, based on the concept of slosh, has been thoroughly investigated in other applications, such as rocket propellants, by groups such as NASA. Ibrahim et al.[11] described slosh as the motion of a given liquid's free surface within a container. As liquid collides with the surface container, energy transfer occurs among the various interfaces. Notably, fluid density must be considered when calculating energy dissipation and motion as it relates to slosh. We suggest that slosh is prevented by taking up compliance inside the skull and bony spinal canal via increased filling of the venous vessels with blood.[24] The reduction in relative motion of the cranial contents when the skull and spinal contents are on the steep part of their compliance curve provides protection from axonal shearing during head trauma, as evidenced by an 83% reduction in measures of axonal injury.[24]

While axonal injury was markedly reduced via IJV compression, it is unclear to what extent this compression alters the response of glial cells to neurological injury following TBI. Glial cells are likely to significantly influence outcome after TBI, as it is well known that astrocytes, oligodendrocytes, and microglia outnumber neurons in humans and play a clear role in maintaining homeostasis and normal function within the brain.[2,5] Therefore, the aim in this study was to examine the effect of venous compression, a potential form of slosh mitigation, on these cells during head injury.

Methods

Tissue samples were obtained from animals studied by us in an earlier analysis.[24] All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of West Virginia University and were performed according to the principles of the *Guide for the Care and Use of Laboratory Animals*.

Experimental Protocol

In brief, 20 male Sprague-Dawley rats weighing between 350 and 400 g were tested. Anesthesia was induced and maintained with isoflurane, and temperature was regulated with a homeothermic heating blanket. The experimental group (10 rats) was fitted with a 15-mm-wide collar with 2 compressive beads designed to overlay the IJVs, which was tightened sufficiently to produce mild compression of the IJVs without compromising the airway. Increases in intraocular pressure and ICP confirmed IJV compression (details described elsewhere).[24] The collar was left in position for 3 minutes prior to inflicting experimental brain injury. The remaining rats constituted the control group (10 rats), and had no collar; otherwise they were the same as the experimental group. All animals were subjected to a standardized acceleration-deceleration head injury model in which a 450-g weight was dropped from a height of 2 m, striking a brass disk that had been surgically affixed to the skull.[8,12]

Tissue Preparation for Histology and Immunohistochemistry

At 7 days postinjury, all animals (20) were anesthetized and immediately perfused transcardially with 200 ml of cold 0.9% saline, followed by an infusion of 4% paraformaldehyde in Millonig buffer for 40 minutes. The entire brain, brainstem, and rostral spinal cord were removed and immediately placed in 4% paraformaldehyde for 24 hours. After 24 hours of fixation, the brain was blocked into 2-mm-thick sections. The first section, from the forebrain, was taken 1.72–3.72 mm from the bregma. The second section, from the cerebellum, was taken 9.12–11.12 mm from the bregma. The resulting tissue was processed using the Tissue-Tek VIP 5 automatic tissue processor (Sakura Finatek) and embedded in paraffin using a Tissue-Tek TEC 5 tissue embedding console system (Sakura Finatek). These embedded tissues were sliced at

a thickness of 6 μm using a Leica RM2235 microtome (Leica Microsystems), and the slices were mounted on glass slides for staining. All slides were heat-fixed and deparaffinized via a series of xylene and alcohol washes prior to immunohistochemical procedures detailed below.

Cresyl Violet. For cresyl violet staining, brain tissue mounted on slides was incubated in 0.1% cresyl violet solution for 10 minutes. After incubation, the tissue was rinsed in deionized H_2O and differentiated in 95% ethyl alcohol for 30 seconds. Following differentiation, the tissue was washed 2 times in xylene prior to coverslip placement. Slides were sealed with acrylic and stored in a laboratory refrigerator.

Hematoxylin and Eosin. Tissue was incubated in hematoxylin (Gill No. 1) for 5 minutes prior to rinsing in deionized water until the water was clear (approximately 5 minutes). Tissue sections were dunked 2–3 times in acid alcohol (1% HCl in 70% EtOH). The sections should be pink after this step. Sections were rinsed for 5 minutes before placing them in ammonia water (1 ml of NH_4OH in 1 L of H_2O), which significantly darkened the sections. Tissues were rinsed in deionized water for 5 minutes and placed in Eosin Y for 1 minute prior to final rinses and coverslip placement.

Fluoro-Jade B. For FJB labeling, the tissue was incubated in 0.06% potassium permanganate for 10 minutes after rehydrating with a series of alcohol and deionized water rinses. Slides were washed for 2 minutes in deionized water prior to a 20-minute incubation in 0.0004% FJB in 0.1% acetic acid. After incubation in the antibody, the tissue was washed 3 times in deionized H_2O and then mounted on slides using an antifade agent. The slides were topped with cover slips, sealed with acrylic, and stored in the dark in a laboratory refrigerator.

Glial Fibrillary Acidic Protein. For GFAP labeling, brain tissue mounted on slides was incubated in polyclonal antibody raised in rabbit against anti–cow GFAP (Dako) at a dilution of 1:500 in 4% horse serum in DPBS overnight. After incubation in the primary antibody, the tissue was washed 3 times in DPBS and incubated in a secondary biotinylated anti–rabbit IgG antibody (Vector Laboratories, Inc.) diluted at 1:10000 in 4% horse serum in DPBS for 4 hours. Tissue was then incubated in horseradish peroxidase–labeled avidin D (Vector Laboratories, Inc.) diluted at 1:1000 in DPBS for 1 hour. Tissue was rinsed 3 times in DPBS and then in diaminobenzidine chromogen solution (Vector Laboratories, Inc.) for 5 minutes. Then the tissue was rinsed in DPBS 3 times and left out to dry overnight. The tissue underwent a final wash in xylene prior to coverslip placement. Slides were then sealed with acrylic and stored in a laboratory refrigerator.

Ionized Calcium Binding Adapter Molecule. For Iba-1 labeling, tissue was processed identically to that for GFAP labeling, with the following exceptions: the primary antibody was rabbit anti–Iba-1 (Wako Chemicals USA) at a dilution of 1:500, and the chromogen solution was NovaRED (Vector Laboratories, Inc.).

Stereological Quantification

A stereological method was used to determine an unbiased estimate of the number of cells expressing each staining marker per cubic mm in the hippocampus, striatum, cortex, and cerebellum. The optical fractionator technique was performed using a Stereo Investigator 9.0 (MBF Bioscience, Inc.) and an Olympus AX70 microscope with 4×–100× objectives. Specimens were examined with low magnification, and regions of interest (volume of tissue) were drawn. The software selected random 75-μm counting frames with a depth of 6 μm, and the object of interest (cell population expressing a given marker) was marked. The volume of the region of interest was determined using the Cavalieri method in which the volume of counting frames was summed along with the number of cells expressing the respective markers. An estimate of the number of cells expressing the protein of interest per cubic millimeter was calculated. All microscopy studies were performed by an observer (Z.J.N.) blinded to the experimental treatment.

Statistical Analysis

Data from all experiments were analyzed using GraphPad Prism 4.0 (GraphPad Software, Inc.). An unpaired t-test was used to compare control animals and experimental animals for FJB-positive neurons, GFAP-positive astrocytes, and Iba-1–positive microglia. A $p < 0.05$ was considered statistically significant for all data analyzed.

Results

Histopathological Analysis

At 1 week after TBI, no gross or histopathological abnormalities were seen in either the IJV compression group or controls. Cresyl violet (Fig. 1) and H & E (Fig. 2) staining revealed no gross contusions or cortical cell loss underlying the impact site, no hippocampal cell loss, and no noticeable difference in striatal or cerebellar integrity.

Neural Degeneration

Fluoro-Jade B, a marker of neural degeneration, was used to visualize and quantify degenerating neurons. A difference between control and IJV compression animals was grossly apparent in all tissue regions visualized (Fig. 3) and was confirmed with stereological quantification (Fig. 4). Statistical analysis comparing control animals with those undergoing IJV compression showed a significant difference in the cortex ($t = 8.522$, $p < 0.0001$), hippocampus ($t = 8.835$, $p < 0.0001$), striatum ($t = 9.439$, $p < 0.0001$), and cerebellum ($t = 6.302$, $p < 0.0001$).

Glial Proliferation

Astrocyte reactivity has been described as both a precursor to neural injury and a marker of neural repair. At 1 week after TBI in the present study, control animals exhibited an increased number of reactive astrocytes at each location assessed using GFAP as a marker, in comparison with the IJV compression animals (Figs. 5 and 6).

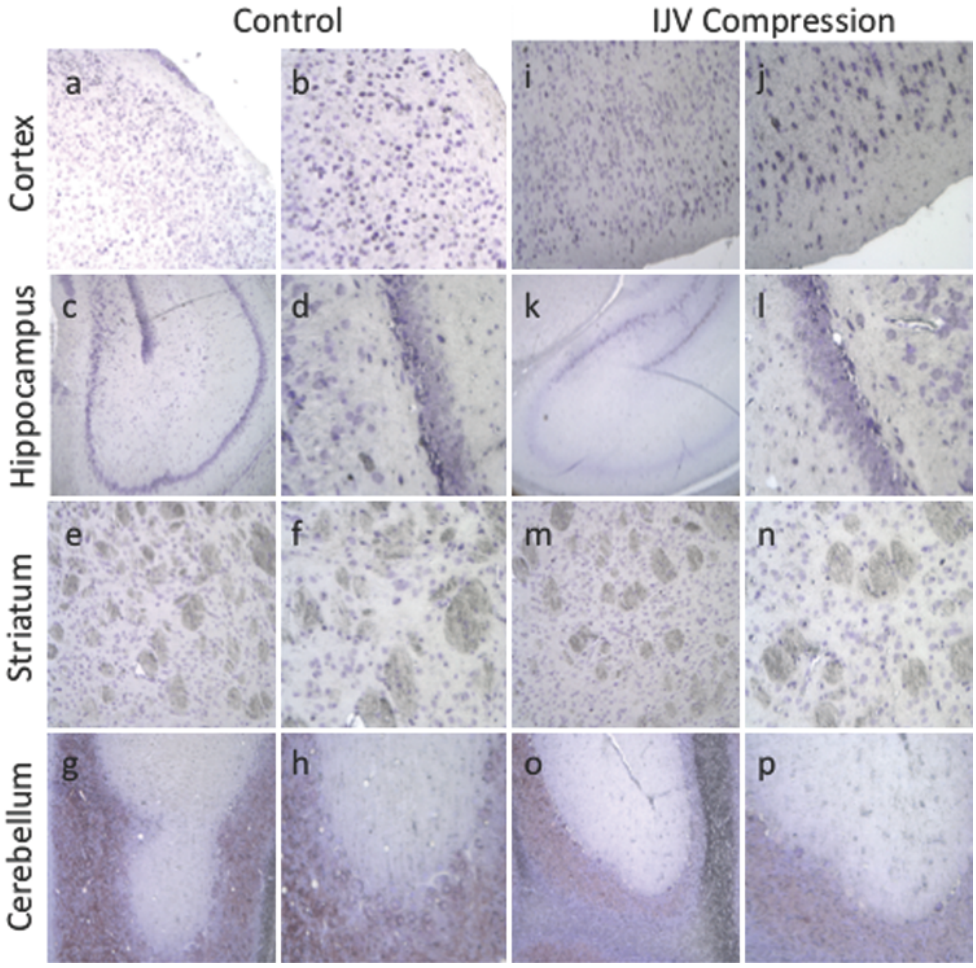

Fig. 1. Cresyl violet staining illustrated no abnormalities following TBI in control animals **(a–h)** or IJV compression animals **(i–p)**. Photomicrographs of low power showing views of the cortex (a and i), hippocampus (c and k), striatum (e and m), and cerebellum (g and o) in control and IJV compression animals, respectively. Original magnification ×10. Photomicrographs of high power showing views of the cortex (b and j), hippocampus (d and l), striatum (f and n), and cerebellum (h and p) in control and IJV compression animals, respectively. Original magnification ×20.

Statistical analysis comparing control animals with those that underwent IJV compression showed a significant difference in the cortex ($t = 8.201$, $p < 0.0001$), hippocampus ($t = 9.334$, $p < 0.0001$), striatum ($t = 8.296$, $p < 0.0001$), and cerebellum ($t = 8.600$, $p < 0.0001$).

Inflammation and Immune Reactivity

Microglial activation following TBI was assessed using Iba-1 as a marker of microglia. At 1 week post-TBI, IJV compression significantly reduced the number of activated microglia at all locations measured (Figs. 7 and 8). Statistical analysis comparing control animals with IJV compression animals showed a significant difference in the cortex ($t = 12.60$, $p < 0.0001$), hippocampus ($t = 9.176$, $p < 0.0001$), striatum ($t = 9.133$, $p < 0.0001$), and cerebellum ($t = 8.979$, $p < 0.0001$).

Discussion

In this study we confirmed the protective effect of IJV compression on neuronal damage. We believe IJV compression leads to slosh mitigation.[24] In our analyses, we used an alternative marker of neural degeneration, FJB,[20] which has been shown to be elevated in numerous TBI models.[1,9,10,19,30] We corroborated these findings by showing an associated marked reduction in posttraumatic glial activation in the compression group. Degenerating neurons were visualized with FJB and were reduced by 48.7%–59.1%, depending on location within the brain. Similarly, reactive astrocytes, which were measured using GFAP, were reduced by 36.8%–45.7%. Microglial activation, indicated by Iba-1 staining, was reduced by 44.1%–65.3%. Notably, activated microglia were seen diffusely throughout the brain, consistent with findings in focal injury models such as controlled cortical impact.[10]

Astrocytes, the most abundant type of glial cell, are essential in maintaining homeostasis in the healthy brain and also play a critical role in the response to injury. Astrocytes have been shown to reduce neuronal death in coculture models of ischemia when compared with neuron-only cultures.[27] The notion of astrocytes facilitating neuronal survival is supported by the observation that the loss of astrocytes after TBI precedes neuronal degeneration.[30] It is believed that astrogliosis, which is usually

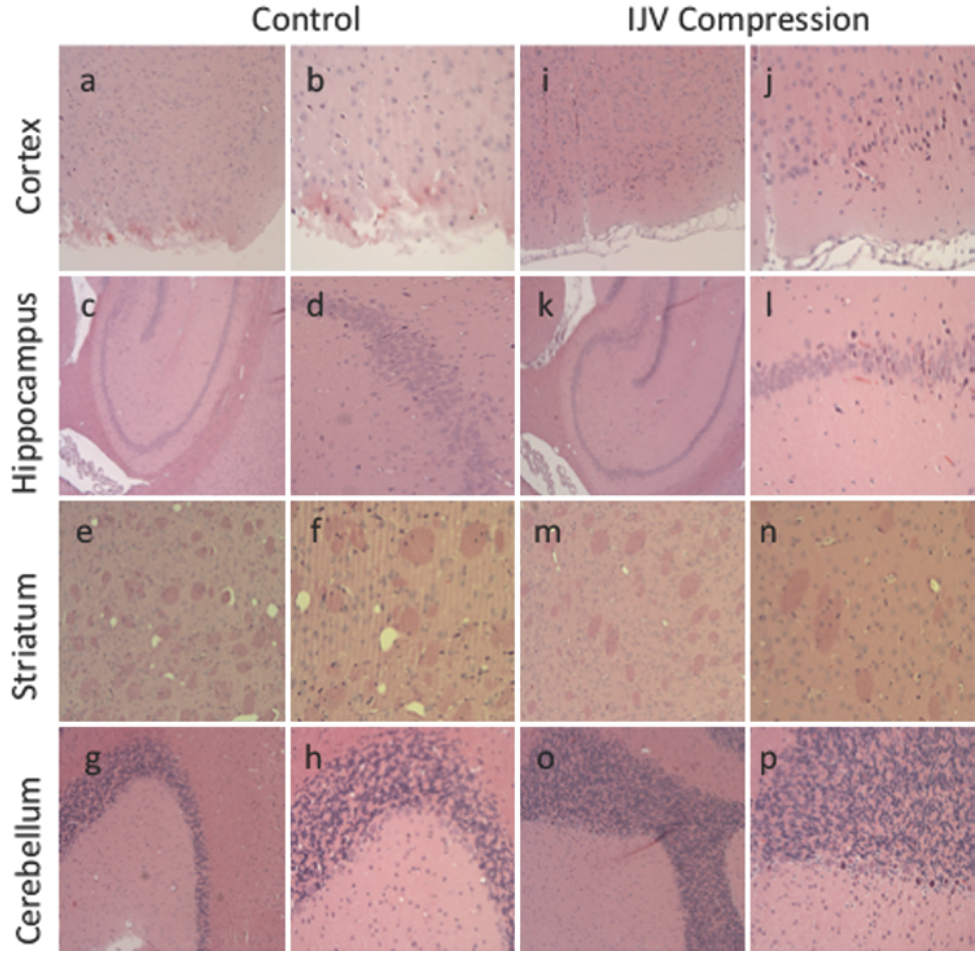

Fig. 2. Hematoxylin and eosin staining showed no abnormalities following TBI in control animals **(a–h)** or IJV compression animals **(i–p)**. Photomicrographs of low power showing views of the cortex (a and i), hippocampus (c and k), striatum (e and m), and cerebellum (g and o) in control and IJV compression animals, respectively. Original magnification ×10. Photomicrographs of high power showing views of the cortex (b and j), hippocampus (d and l), striatum (f and n), and cerebellum (h and p) in control and IJV compression animals, respectively. Original magnification ×20.

initiated shortly after CNS injury, is involved in the process of restoring CNS homeostasis.[7] In excess, however, astrogliosis may lead to glial scarring, which can impede neural repair and contribute to axonal degeneration.[17]

Similarly, microglia are capable of secreting both neurotrophic and neurodegenerative factors.[18,23,26] While it is unclear whether microglial activation precedes or is a consequence of axonal injury, it is known to occur in both the acute and chronic phases after TBI and is therefore a suitable marker for neurological injury.[23]

Translating to Humans

While experimental results are promising, questions remain concerning the translation of our findings to the clinical population. Perhaps most importantly, can IJV compression produce similar physiological changes in humans? The Queckenstedt maneuver, in which the clinician applies pressure over the IJVs while measuring ICP, indicates that it is possible to produce transient changes in ICP with IJV manipulation. Other questions regarding how long this response can be maintained, what compensatory changes may be induced, and what functional impairments or discomfort will be produced remain to be addressed.

Study Limitations

We have previously demonstrated an ability to alter intracranial physiology through IJV compression using surrogate markers of cerebral volume such as intraocular pressure and ICP. We believe the increase in cerebral volume may reduce the propensity for brain slosh. To truly confirm such a reduced propensity, further studies in which imaging is performed to assess cerebral volume are needed.

The Marmarou version of acceleration-deceleration injury used in the present work is a well-studied model producing axonal injury through the initial impact, as well as the subsequent rebound injury, since the animal is placed on a foam pad with a known spring constant.[12] As such, alterations in injury biomechanics can produce changes in injury severity. The collar used in the present study to produce IJV compression did not alter injury

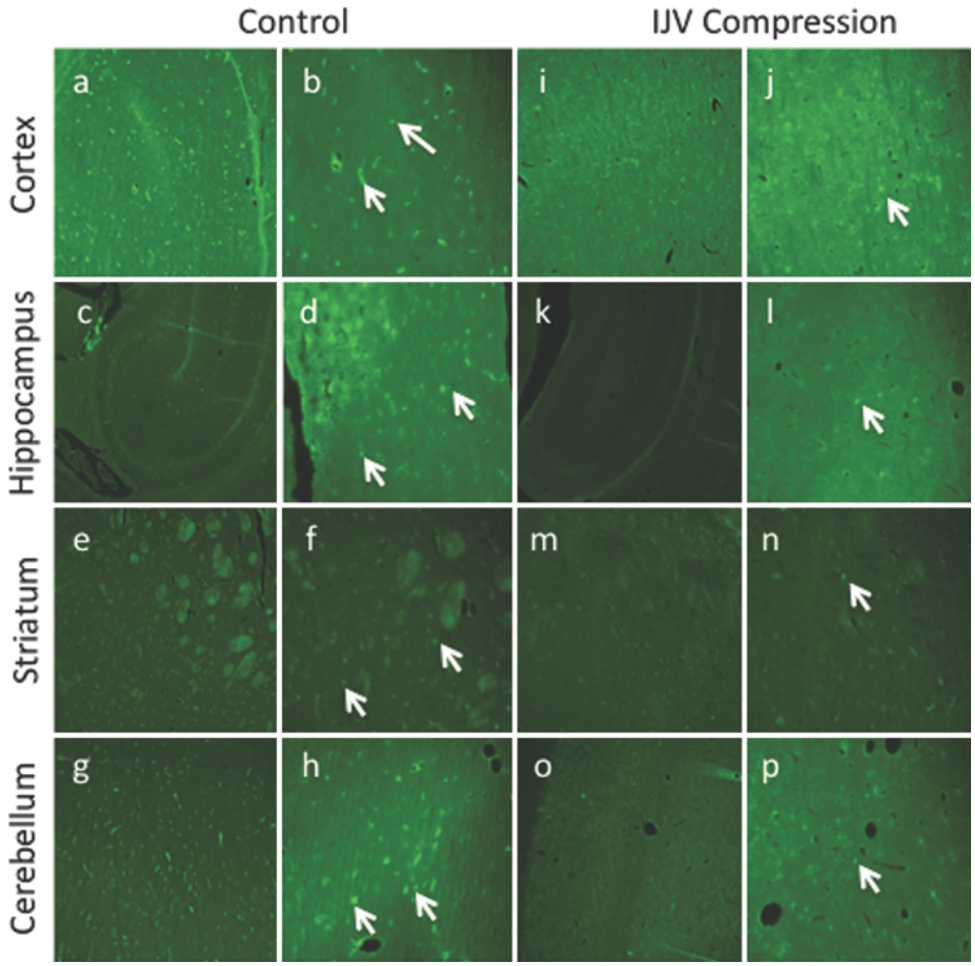

Fig. 3. Fluoro-Jade B staining showed a significant reduction in degenerating neurons in IJV compression animals **(i–p)** compared with controls **(a–h)**. Photomicrographs of low power showing views of the cortex (a and i), hippocampus (c and k), striatum (e and m), and cerebellum (g and o) in control and IJV compression animals, respectively. Original magnification ×10. Photomicrographs of high power showing views of cortex (b and j), hippocampus (d and l), striatum (f and n), and cerebellum (h and p) in control and IJV compression animals, respectively. Original magnification ×20. *Arrows* indicate degenerating neurons.

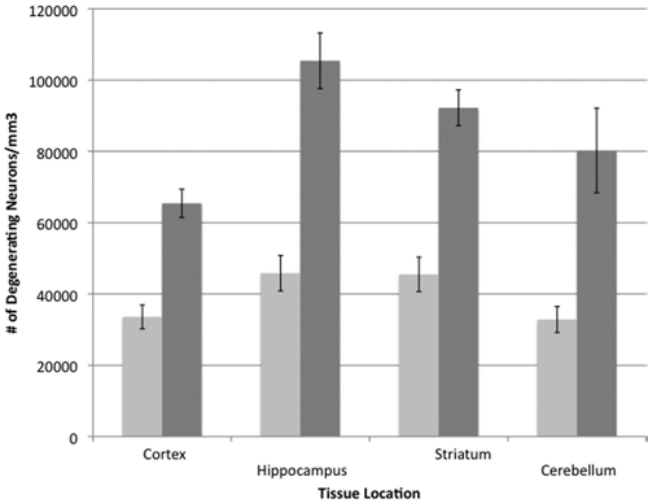

Fig. 4. Bar graph demonstrating stereological quantification of degenerating neurons stained with FJB. A significant reduction in neural degeneration was seen in IJV compression animals *(light gray bars)* at all tissue locations visualized and quantified ($p < 0.0001$). *Dark gray bars* represent control animals.

biomechanics, consistent with the fact it is composed of an elastic band with compressive beads. The beads were required to provide compression without airway compromise. Control animals were not fitted with any form of collar because of the potential for inadvertent compression or twisting associated with even a noncompressive collar.

Despite demonstrating a significant reduction in multiple markers of neural injury, we did not assess the effects on neurological outcome in this study. Future studies are needed to address the effect of IJV compression before TBI on neurological outcome, particularly aspects such as memory or cognition and changes in affect, which are often of clinical interest following TBI.

Conclusions

Our findings suggest that IJV compression as well as the associated slosh mitigation may be a powerful technique for reducing TBI. This technique reduces not only the axonal injury, but also the number of degenerating neurons, reactive astrocytes, and activated microglia.

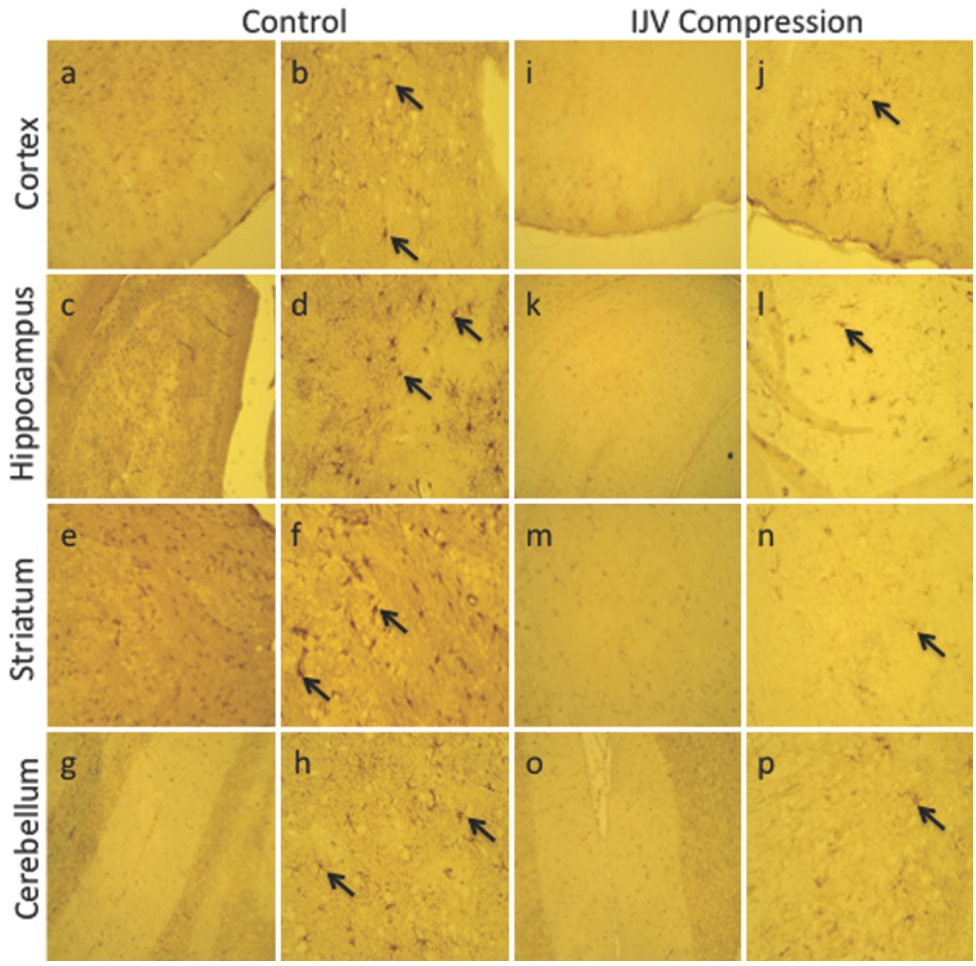

Fig. 5. Glial fibrillary acidic protein staining showed a significant reduction in reactive astrocytes in IJV compression animals **(i–p)** compared with controls **(a–h)**. Photomicrographs of low power showing views of cortex (a and i), hippocampus (c and k), striatum (e and m), and cerebellum (g and o) in control and IJV compression animals, respectively. Original magnification ×10. Photomicrographs of high power showing views of cortex (b and j), hippocampus (d and l), striatum (f and n), and cerebellum (h and p) in control and IJV compression animals, respectively. Original magnification ×20. *Arrows* indicate reactive astrocytes.

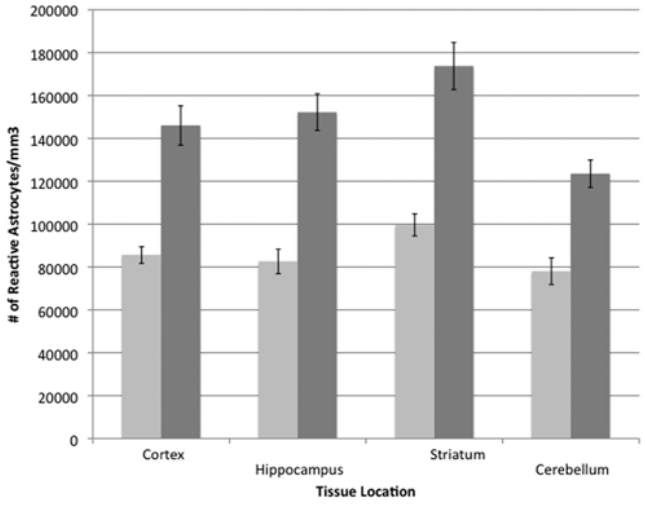

Fig. 6. Bar graph demonstrating stereological quantification of reactive astrocytes stained with GFAP. A significant reduction in reactive astrocytes was seen in IJV compression animals *(light gray bars)* at all tissue locations visualized and quantified ($p < 0.0001$). *Dark gray bars* represent the control animals.

While axonal injury is a well-documented pathological finding after TBI, and one for which current preventative techniques are inadequate, injury to neurons and associated axons hardly exists in isolation. Rather than taking a neuron-centered approach to TBI prevention and treatment, the neuron-glial interaction should probably be considered. Microglia and astrocytes are intimately involved in the initial injury process and subsequent inflammation as well as the long-term repair and recovery that occur. We believe that IJV compression, and presumably slosh mitigation, offers a novel form of TBI prevention and addresses the intracranial environment more effectively than extracranial protective devices such as helmets. Internal jugular vein compression probably leads to increased retention of blood in the cranium and may alter the density gradients among structures, reducing energy displacement in brain tissue based on the concept of slosh in fluid dynamics. More research is needed to verify that our initial findings can translate into clinical applications and provide benefit based on various TBI clinical outcome measures.

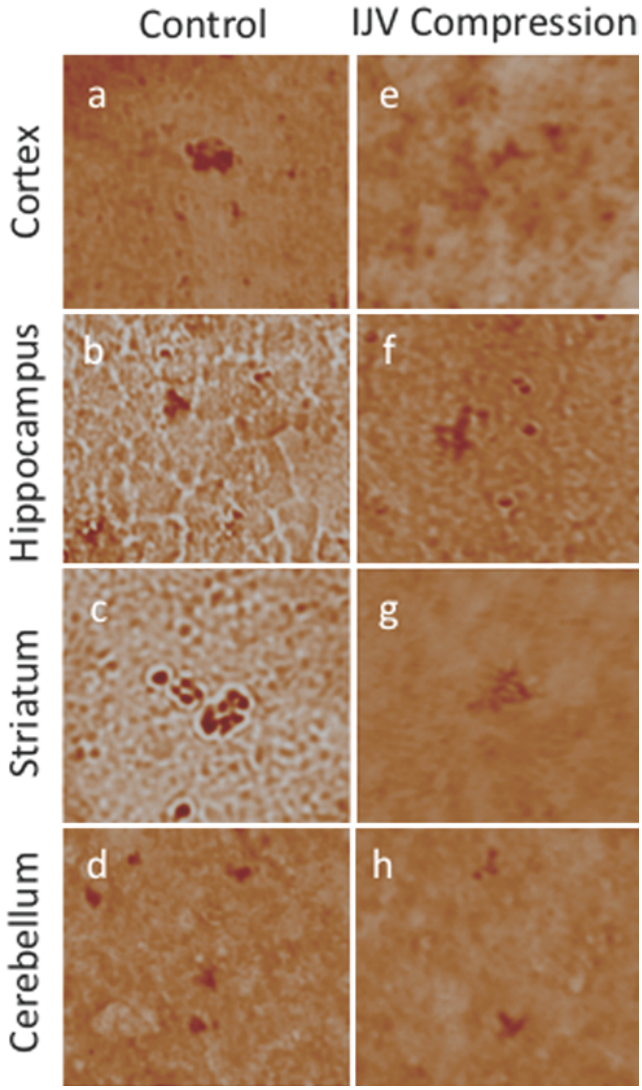

Fig. 7. Staining for Iba-1 showed a significant reduction in activated microglia in IJV compression animals **(e–h)** compared with controls **(a–d)**. Photomicrographs of high power showing views of the cortex (a and e), hippocampus (b and f), striatum (c and g), and cerebellum (d and h) in control and IJV compression animals, respectively. Original magnification ×40.

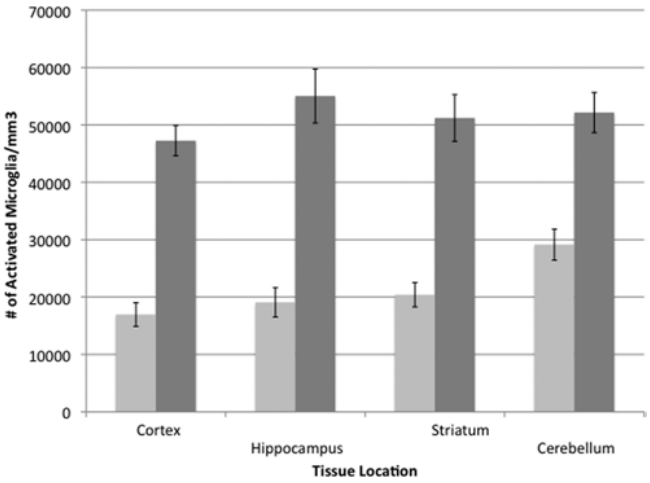

Fig. 8. Bar graph demonstrating results of stereological quantification of activated microglia stained with Iba-1. A significant reduction in activated microglia was seen in IJV compression animals *(light gray bars)* at all tissue locations visualized and quantified ($p < 0.0001$). *Dark gray bars* represent the control animals.

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Disclosure

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Dr. Smith is stockholder and intellectual property owner with Traumatic Brain Injury Innovations. Dr. Fisher receives royalties for licensing-related technology from Traumatic Brain Injury Innovations.

Author contributions to the study and manuscript preparation include the following. Conception and design: Rosen, Turner, Smith, Fisher. Acquisition of data: Turner, Naser. Analysis and interpretation of data: Rosen, Turner. Drafting the article: Turner, Naser. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Rosen. Statistical analysis: Turner. Administrative/technical/material support: Bailes. Study supervision: Rosen, Turner, Bailes.

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