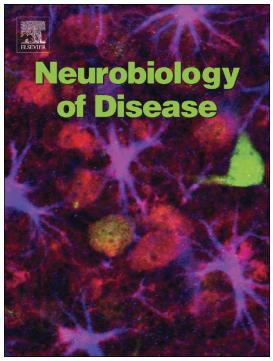

Crotalus helleri venom preconditioning reduces postoperative cerebral edema and improves neurological outcomes after surgical brain injury

Cherine H. Kim, Devin W. McBride, Prativa Sherchan, Carl E. Person, Eric C.K. Gren, Wayne Kelln, Tim Lekic, William K. Hayes, Jiping Tang, John H. Zhang

PII: S0969-9961(17)30049-9
DOI: doi: 10.1016/j.nbd.2017.03.001
Reference: YNBDI 3915

To appear in: *Neurobiology of Disease*

Received date: 3 May 2016
Revised date: 12 December 2016
Accepted date: 8 March 2017

Please cite this article as: Cherine H. Kim, Devin W. McBride, Prativa Sherchan, Carl E. Person, Eric C.K. Gren, Wayne Kelln, Tim Lekic, William K. Hayes, Jiping Tang, John H. Zhang , Crotalus helleri venom preconditioning reduces postoperative cerebral edema and improves neurological outcomes after surgical brain injury. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Ynbdi(2017), doi: 10.1016/j.nbd.2017.03.001

Crotalus helleri venom preconditioning reduces postoperative cerebral edema and improves neurological outcomes after surgical brain injury.

Cherine H. Kim, MD, PhD,[a] Devin W. McBride, PhD,[a] Prativa Sherchan, PhD,[a] Carl E. Person,[b] Eric C.K. Gren, PhD,[b] Wayne Kelln, PhD,[b] Tim Lekic, MD, PhD,[a,e] William K. Hayes, PhD,[b] Jiping Tang, MD,[a] and John H. Zhang, MD, PhD[a,c,d]

[a]Department of Physiology & Pharmacology, [b]Department of Earth and Biological Sciences, [c]Department of Neurosurgery, [d]Department of Anesthesiology, [e]Department of Neurology, Loma Linda University School of Medicine, Loma Linda, California, USA

Corresponding author:

John H. Zhang, MD, PhD

Department of Physiology & Pharmacology

Loma Linda University School of Medicine

Loma Linda, CA 92350

Phone: (909) 558-4723; *Fax:* (909) 558-0119; *Email:* jhzhang@llu.edu

Running head: *C. helleri* preconditioning reduces postoperative cerebral edema

Funding support was provided by NIH grant R01NS084921 to John H. Zhang.

Conflict of Interest Statement: The authors have declared that no conflict of interest exists

ABSTRACT

Introduction: Postoperative cerebral edema is a devastating complication in neurosurgical patients. Loss of blood-brain barrier integrity has been shown to lead to the development of brain edema following neurosurgical procedures. The aim of this study was to evaluate preconditioning with *Crotalus helleri* venom (Cv-PC) as a potential preventive therapy for reducing postoperative brain edema in the rodent SBI model. *C. helleri* venom is known to contain phospholipase A2 (PLA2), an enzyme upstream to cyclooxygenase-2 (COX-2) in the inflammatory cascade, acts to increase the production of inflammatory mediators, such as prostaglandins. We hypothesize that Cv-PC will downregulate the response of the COX-2 pathway to injury, thereby reducing the inflammatory response and the development of brain edema after SBI.

Materials and Methods: 75 male Sprague Dawley rats (280-330g) were divided to the following groups—naïve+vehicle, naïve+Cv-PC, sham, vehicle, Cv-PC, Cv-PC+NS398 (COX-2 inhibitor). Vehicle preconditioned and Cv-PC animals received either three daily subcutaneous doses of saline or *C. helleri* venom at 72 h, 48 h, and 24 h prior to surgery. In Cv-PC+NS398 animals, NS398 was administered intraperitoneally one hour prior to each Cv-PC injection. Sham-operated animals received craniotomy only, whereas SBI animals received a partial right frontal lobectomy. Neurological testing and brain water content were assessed at 24 h and 72 h after SBI; COX-2 and PGE_2 expression was assessed at 24 h postoperatively by Western blot and immunohistochemistry, respectively.

Results: At 24 h after SBI, the vehicle-treated animals were observed to have increased brain water content (83.1 ± 0.2%) compared to that of sham animals (80.2 ± 0.1%). The brain water content of vehicle-treated animals at 72 h post-SBI was elevated at 83.3 ± 0.2%. Cv-PC-treated animals with doses of 10% LD_{50} had significantly reduced brain water content of 81.92 ± 0.7% and 81.82 ± 0.3% at 24 h and 72 h, respectively, after SBI compared to that of vehicle-treated animals, while Cv-PC with 5% LD_{50} doses showed brain water content that trended lower but did not reach statistical significance. At 24 h and 72 h post-SBI, Cv-PC-treated animals had significantly higher neurological score than vehicle-treated animals. The COX-2 over-expression characterized in SBI was attenuated in Cv-PC-treated animals;

NS398 reversed the protective effect of Cv-PC on COX-2 expression. Cv-PC tempered the over-expression of the inflammatory marker PGE_2.

Conclusion: Our findings indicate that Cv-PC may provide a promising therapy for reducing postoperative edema and improving neurological function after neurosurgical procedures.

Keywords: Crotalus helleri, rattlesnake venom, surgical brain injury, brain edema, cerebral edema, preconditioning

Abbreviations: Cv-PC, *Crotalus helleri* rattlesnake venom preconditioning; COX-2, cyclooxygenase-2; PLA2, phospholipase A2; PGE_2, prostaglandin E2; SBI, surgical brain injury

INTRODUCTION

The delicate and complex architecture of the brain presents significant challenges for neurosurgery. To date, developing less invasive surgical methods (Decq et al. 1998, Gerzeny and Cohen 1998) and administering nonspecific postoperative care (Bruder and Ravussin 1999, Hellwig et al. 2003) have been the predominant strategies for limiting surgical brain injury (SBI). However, relatively little research exists that focuses on understanding SBI's pathophysiology or developing therapies to target specific pathways. Previous studies have established that brain edema develops several hours after SBI and is likely the result of compromised BBB integrity (Matchett et al. 2006, Jadhav et al. 2007a, Jadhav et al. 2007b). SBI-induced brain edema requires vigilant postoperative care and may lengthen hospital stay. Thus, reducing brain edema caused by SBI would have a significant impact on both patient outcome and perioperative costs.

The elective nature of many neurosurgical procedures makes SBI a prime candidate for preventative therapy. Preconditioning (PC) utilizes normally harmful methods which, when given well-below toxic levels, induce minimal damage to elicit the body's innate response and reduce damage from the full-insult. While PC studies have shown promising neuroprotective effects for other models of brain injury (Wada et al. 1996, Kapinya et al. 2002), clinical translation is limited in these models since these injuries develop spontaneously. Currently, hyperbaric oxygen preconditioning (HBO-PC) is the only preconditioning modality that has been studied in animal model of SBI. HBO-PC was shown to decrease brain water content and improve neurological function 24 h following SBI; these effects were eradicated with cyclooxygenase-2 (COX-2) inhibition (Jadhav et al. 2009). Furthermore, SBI-induced COX-2 overexpression was tempered by HBO-PC, highlighting the PLA_2/COX-2 pathway as promising target for therapy.

Snake venom toxins have long been studied for potential therapeutic applications. *Crotalus helleri* venom contains phospholipase 2 (PLA_2), an enzyme upstream to COX-2 in the inflammatory cascade, that converts membrane phospholipids into arachidonic acid, which eventually leads to the production of prostaglandins and thromboxanes (Bush and Siedenburg 2000; French et al. 2004). We

hypothesize that Cv-PC will downregulate the response of the PLA_2/COX-2 pathway to injury, thereby reducing the inflammatory response after SBI.

MATERIALS AND METHODS

Animals

All animal protocols followed NIH Guide for Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee at Loma Linda University. Seventy-five male Sprague Dawley rats (280-330 g) were used. Animals were housed in cages on a constant 12-hour light/dark cycle with controlled temperature and were given food and water ad libitum.

Experimental Groups, Preconditioning Regimen, and Interventions

The rats were divided into following five groups—Naïve+vehicle (n=1), Naïve+Cv-PC (n=3), Sham (n=13), SBI+vehicle (n=21), SBI+Cv-PC (n=26), and SBI+Cv-PC+NS398 (n=11).

Rats chosen for SBI were preconditioned with either the vehicle (normal saline) or *C. helleri* venom (Cv-PC). The preconditioning regimen was as follows: a single subcutaneous injection of either the vehicle or *C. helleri* venom was given each day for three consecutive days. The last day of preconditioning was 24 hours before SBI.

In the dose study (Figure 1), *C. helleri* venom was administered at 5% or 10% LD_{50}. For the remainder of the SBI experiments, the 10% LD_{50} dose of *C. helleri* venom was used. In the group of SBI+Cv-PC rats which received the COX-2 inhibitor NS398 (Jadhav et al. 2009), on each day of Cv-PC, NS398 (10 mg/kg, normal saline) was administered by intraperitoneal injection 1 h prior to venom administration. The COX-2 inhibitor NS398 was purchased from Abcam (Cambridge, MA).

C. helleri crude venom was collected, by voluntary extraction into a parafulm-covered beaker, from a *C. helleri* which is housed in Dr. Hayes' laboratory. All *C. helleri* venom used throughout this study was collected from the same snake and from consecutive milkings. After milking, the venom was

lyophilized and then stored at -20°C. Lyophilized venom was reconstituted in normal saline to the working doses (5%, 10%, 25%, and 50% LD_{50}). The LD_{50} dose for *C. helleri* venom via subcutaneous injection is 3.65 mg/kg (Minton 1956). Thus, the mass concentrations of 5%, 10%, 25%, and 50% LD_{50} are 0.18 mg/kg, 0.36 mg/kg, 0.71 mg/kg, and 1.83 mg/kg, respectively.

SBI Rat Model

The SBI rat model was made by performing a partial resection of the right frontal lobe as previously described (Matchett et al. 2006; Jadhav et al. 2007a, Jadhav et al. 2007b). Rats were anesthetized with isoflurane (4% induction and 2.5% maintenance) delivered with 70% medical air and 30% oxygen. A midline skin incision was made and periosteum was reflected to expose the skull. A cranial window was made in the right frontal bone with margins 2 mm lateral to the sagittal suture and 1 mm proximal to the coronal suture to expose the dura. The dura was incised and underlying frontal lobe was resected following margins of the cranial window. Hemostasis was ensured with normal saline irrigation and intra-operative packing after which the skin was sutured. Sham animals were subjected to the same procedures but frontal lobe was left intact. Animals were closely observed for post-operative recovery and then transferred to their home cages.

Neurological Testing

Neurological testing was performed by a blinded examiner at 24 h after surgery. A 21-point composite neuroscore test was used to evaluate sensorimotor function, as previously described (McBride et al. 2015). Briefly, seven parameters were tested including spontaneous activity, body proprioception, response to vibrissae touch, limb symmetry, lateral turning ability, forepaw outstretching, and climbing ability, with a maximum of 3 points designated for each category.

Brain Water Content

After neurological testing, animals were euthanized with isoflurane and sacrificed at 24 h for brain harvesting. Brains were divided into ipsilateral and contralateral frontal and parietal lobes, cerebellum, and brainstem. These sections were weighed immediately (wet weight), then placed in a 100 degree Celsius oven for 48 h before being weighed again (dry weight). Brain water content was calculated as a percentage via the following formula: [(wet weight – dry weight)/(wet weight)] x 100% (Tang et al. 2004, Sherchan et al. 2016).

Western Blot

At 24 h after SBI, animals were euthanized with isoflurane and perfused transcardially with cold PBS. Brains were divided into hemispheres and stored at -80°C until analysis. Whole-cell lysates were obtained by gently homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Inc., sc-24948) and centrifuging (14,000 g at 4°C for 30 min). The supernatant was collected and the protein concentration was determined using a detergent compatible assay (Bio-Rad, Dc protein assay). Equal amounts of protein (30 μg) were loaded and subjected to electrophoresis on an SDS-PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was blocked and incubated with the primary antibody overnight at 4°C. For the primary antibody goat polyclonal to COX-2 1/500 (Santa Cruz, SC-1745) was used. The same membrane was probed with an antibody against β-actin (Santa Cruz, 1:1000) for an internal control. Incubation with secondary antibodies (Santa Cruz Biotechnology) was done for 1 h at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL) and visualized with an imaging system (Bio-Rad, Versa Doc, model 4000). Data was analyzed using Image J software.

Immunohistochemistry

The coronal sections (10 μm thickness) containing the bilateral frontal lobes were cut on a cryostat (Leica Microsystems, Bannockburn, IL) and mounted on poly-L-lysine–coated slides. Sections were incubated overnight at 4°C with goat polyclonal antibody to COX-2 (Santa Cruz, SC-1745). Appropriate fluorescence dye–conjugated secondary antibodies (Jackson Immunoresearch, West Grove,

PA) were applied in the dark for 1 hour at room temperature. For negative controls, the primary antibodies were omitted and the same staining procedures were performed. The sections were visualized with a fluorescence microscope, and the photomicrographs were saved and merged with Image Pro Plus software (Olympus, Melville, NY).

Local Skin Inflammation

Four naïve rats were used to examine local inflammation at the injection site (subcutaneous in the right haunch). These animals received a subcutaneous injection on three consecutive days. Twenty-four hours after the final injection, the rats were euthanized. The right haunches were imaged, and then the skin was cut to expose the subcutaneous region of the injection site, and another image was taken. Animals were injected with either normal saline (n=1) or *C. helleri* venom (at doses of 10%, 25%, or 50% LD_{50}) (n=1/Cv-PC).

Statistical Analysis

All values are mean ± SEM. GraphPad Prism software was used for statistical analysis. Brain water content and Western blot data was analyzed using one-way ANOVA with Tukey's comparisons. Neurological function (Garcia Score) was analyzed using ANOVA on Ranks. A P value <0.05 was considered significant.

RESULTS

The mortality in each group is as follows: 0/13 (or 0%) for sham-operated rats, 3/21 (or 14%) for vehicle preconditioned SBI rats, 2/26 (or 8%) for Cv-PC SBI rats, and 1/11 (or 9%) for SBI rats preconditioned with NS398+Cv-PC.

Cv-PC Reduces Brain Water Content after SBI

At 24 h after SBI, the vehicle preconditioned animals were observed to have increased right frontal lobe brain water content of 83.1 ± 0.2%, which was significantly higher than that of sham animals, whose brain water content was 80.2 ± 0.1% (Figure 1B). Similarly, the brain water content of vehicle preconditioned animals at 72 h post-SBI was elevated at 83.3 ± 0.2% (Figure 1C). Cv-PC animals with doses of 10% LD_{50} had significantly reduced brain water content of 81.92 ± 0.3% and 81.82 ± 0.1% at 24 h and 72 h, respectively, after SBI compared to that of vehicle preconditioned animals, while Cv-PC with 5% LD_{50} showed brain water content that trended lower but did not reach statistically significance compared to vehicle preconditioned animals.

Very few differences were observed between any group for the brain water content of the right parietal lobe, left frontal lobe, left parietal lobe, cerebellum, and brain stem. At 24 h post-SBI, the left frontal lobe had a significantly lower brain water content for SBI animals receiving Cv-PC with 5% LD_{50} compared to that of sham animals. At 72 h post-SBI, SBI animals preconditioned with the vehicle had a significantly higher brain water content in the right parietal lobe compared to that of sham animals.

Cv-PC Improves Neurological Function after SBI

To assess the neurological function after Cv-PC, the modified Garcia neurological scores were assessed (Figure 1D and E). All animals that received SBI scored significantly lower than sham animals (20.5 ± 0.2). At 24 h and 72 h post-SBI, Cv-PC animals receiving 10% LD_{50} venom (18.3 ± 0.3 and 18.2 ± 0.4, respectively) scored significantly higher than vehicle preconditioned animals (16.4 ± 0.4 and 15.8 ± 0.6, respectively). The Cv-PC with 5% LD_{50} did not improve neurological function at 24 h after SBI.

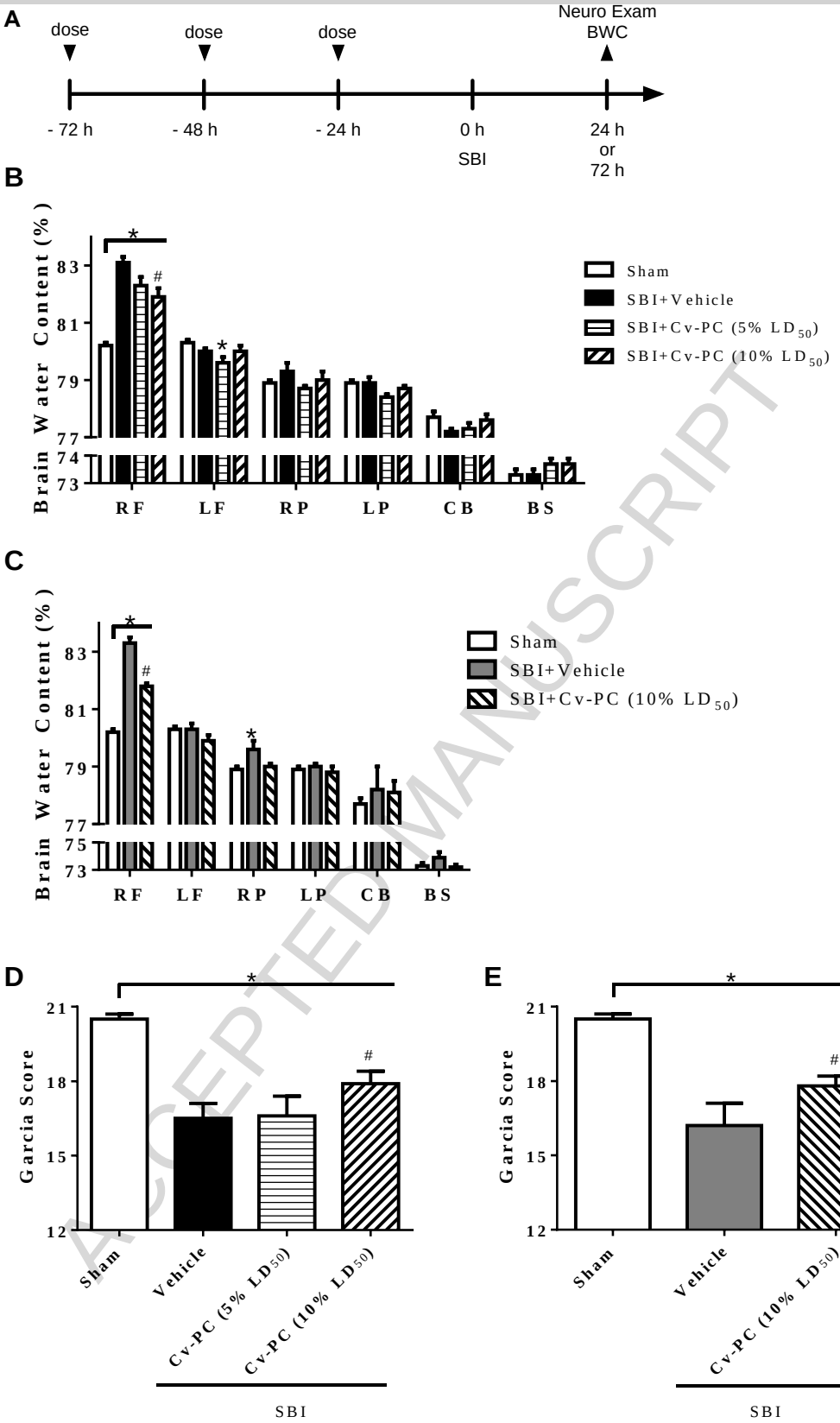

Figure 1. Cv-PC reduces brain edema and improves neurological function 24 and 72 h after SBI. (A) Schematic timeline of the dosing study. (B) Brain water content for the right frontal lobe (RF), left frontal lobe (LF), right parietal lobe (RP), left parietal lobe (LP), cerebellum (CB), and brain stem (BS) 24 h after SBI. (C) Brain water content 72 h after SBI. (D) Neurological function 24 h after SBI. (E) Neurological function 72 h after SBI. * $p<0.05$ vs Sham # $p<0.05$ vs SBI+Vehicle. Data are shown as mean ± SEM. n=5-7 all groups.

COX-2 Inhibition Reverses Cv-PC Effects on Brain Water Content and Neurological Function after SBI

To evaluate the role of COX-2 in Cv-PC against SBI, NS398, a COX-2 inhibitor, was administered one hour prior to each Cv-PC dose. This abolished the decrease in the right frontal lobe brain water content by Cv-PC (Figure 2B) to levels comparable to vehicle preconditioned animals. No differences were observed between any groups for the right parietal lobe, left frontal and parietal lobes, and brain stem. An increased brain water content of the cerebellum was observed for the SBI animals given NS398 with Cv-PC compared to SBI animals preconditioned with vehicle.

Similar to the dosing study, the neurological function of SBI animals preconditioned with the vehicle were significant compared to sham animals, and Cv-PC lead to functional recovery. COX-2 inhibition by NS398 during Cv-PC reversed the protective effects of Cv-PC on neurological function at 24 h post-SBI (Figure 2C).

Cv-PC Reduces Expression of Inflammatory Mediators COX-2 and PGE_2 after SBI

At 24 h following SBI, levels of COX-2 were increased in vehicle-treated animals, as expected. The expression of COX-2 was significantly reduced in Cv-PC-treated animals. COX-2 inhibition during Cv-PC treatment abolished this protective effect but provoked COX-2 expression at 24 h post-SBI (Figure 3). To assess the effects of Cv-PC on downstream inflammatory mediators, PGE_2 was evaluated by immunohistochemistry. Cv-PC appeared to diminish the expression of PGE_2 at 24 h following SBI compared to that of vehicle-treated animals (Figure 4).

Cv-PC Causes Minimal Local Inflammation in Naïve Rats

At 24 h following the last dose of Cv-PC, preconditioning with 10% LD_{50} causes no apparent local inflammation on the epidermis or the subcutaneous region of the injection site. The epidermis and subcutaneous region at the injection site for the vehicle preconditioned animals and the rats receiving Cv-PC with 10% LD_{50} are indistinguishable (Figure 5A-D). However, local inflammation was observed for

Cv-PC with 25% LD_{50} which is indicated by marked redness and swelling on the subcutaneous region of the skin (Figure 5F); no apparent inflammation is observed on the epidermis of Cv-PC with 25% LD_{50} (Figure 5E). Cv-PC with 50% LD_{50} lead to even greater subcutaneous inflammation, as indicated by swelling, redness, and hemorrhage (Figure 5H), and even showed signs of bruising on the epidermis (Figure 5G).

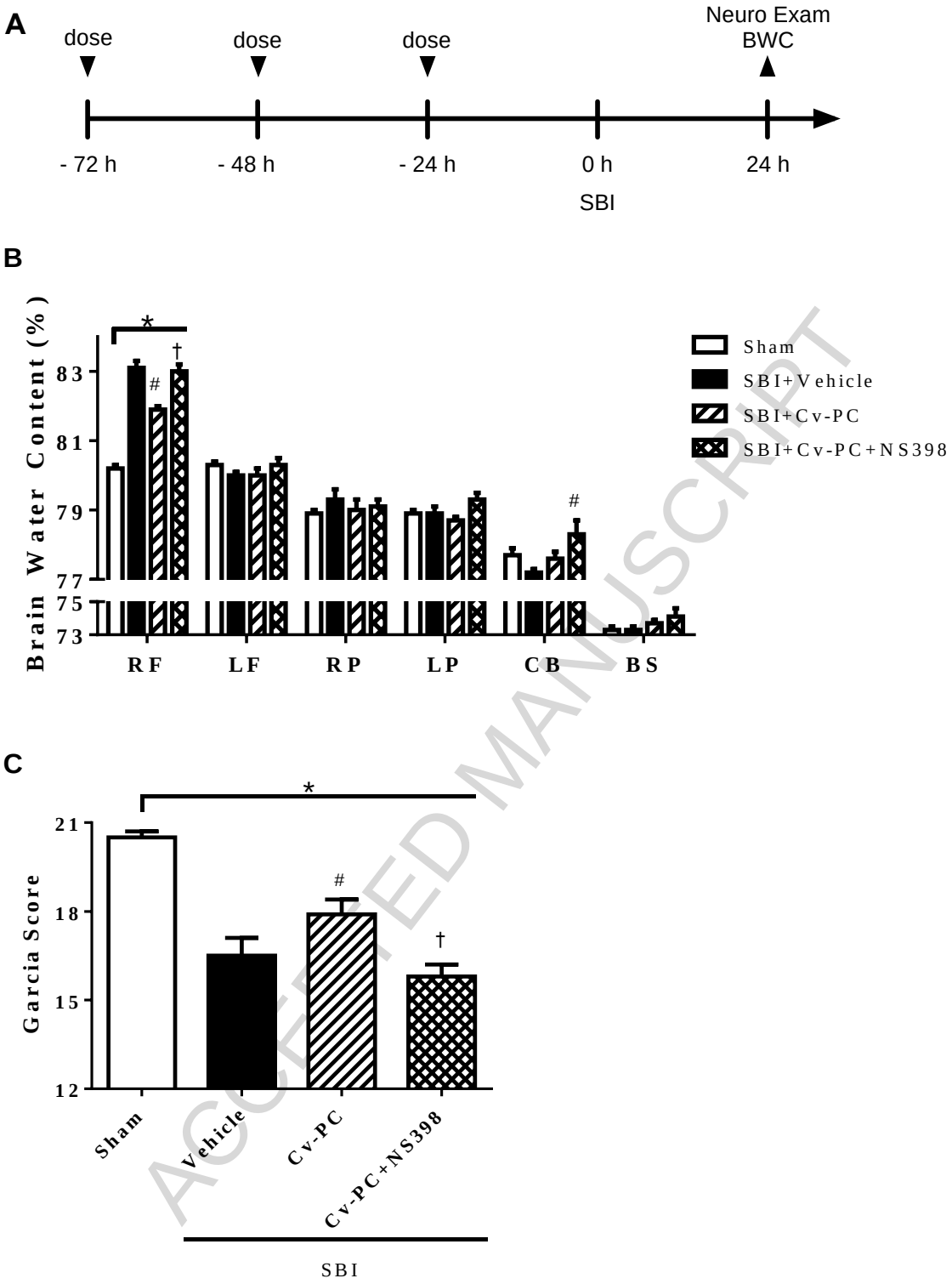

Figure 2. COX-2 Inhibition Reverses Cv-PC Effects. (A) Schematic timeline of injections, surgery, and outcomes. (B) Effect of NS398 (COX-2 inhibitor) and Cv-PC on the brain water content for the right frontal lobe (RF), left frontal lobe (LF), right parietal lobe (RP), left parietal lobe (LP), cerebellum (CB), and brain stem (BS) 24 h after SBI. (C) Effect of NS398 (COX-2 inhibitor) and Cv-PC on neurofunctional recovery 24 h after SBI. * $p<0.05$ vs Sham, # $p<0.05$ vs SBI+Vehicle, † $p<0.05$ vs SBI+Cv-PC. Data are shown as mean ± SEM. n=5-7 all groups.

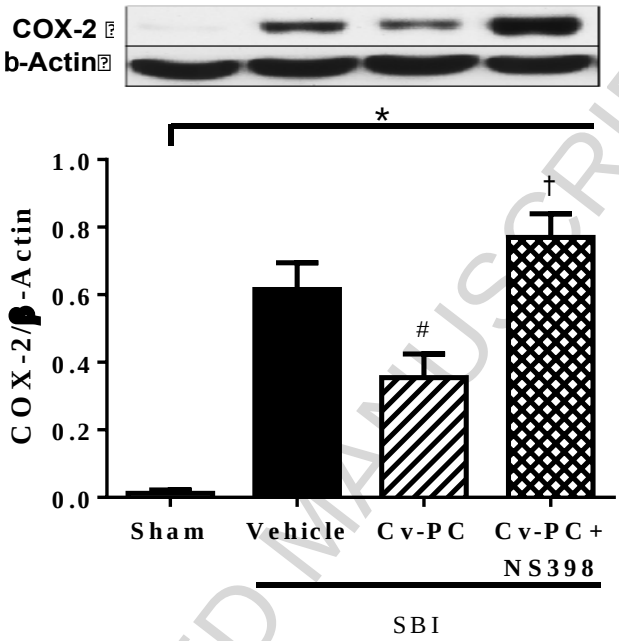

Figure 3. COX-2 Western Blot Analysis. Brain expression of COX-2 was significantly decreased with Cv-PC at 24 h after surgical brain injury. Injection of NS398, a COX-2 inhibitor, 1 h prior to Cv-PC reversed this effect. * $p<0.05$ vs Sham, # $p<0.05$ vs Vehicle, † $p<0.05$ vs SBI+Cv-PC. Data are shown as mean ± SEM. n=5/group.

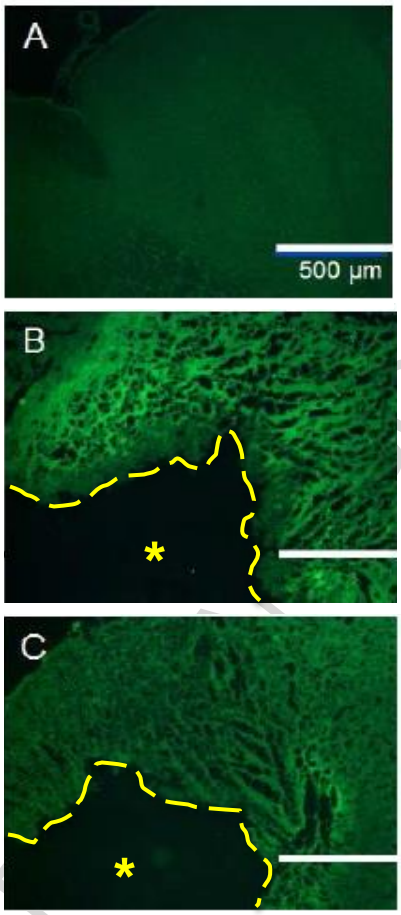

Figure 4. Immunohistological staining of an axial slice of the ipsilateral frontal lobe near the resection site using inflammatory marker PGE_2 (green) for Sham (A), SBI+Vehicle (B), and SBI+Cv-PC (C). The resection margin is outlined with the yellow dashed line and the resection cavity is denoted by the asterisk (no outline in A since this is Sham). Scale bar = 500 µm. n=2/group.

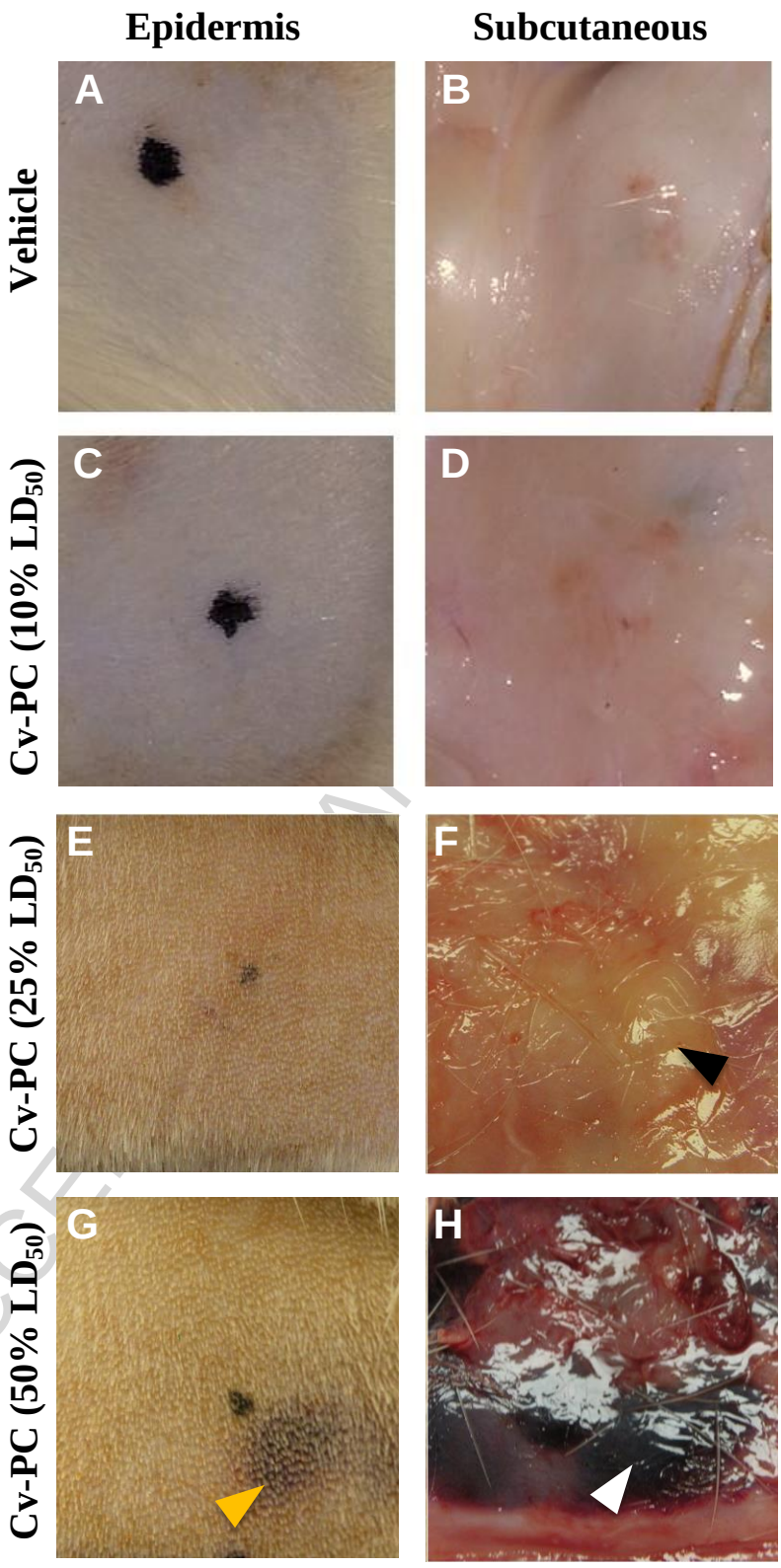

Figure 5. Gross anatomical images of the epidermis (left images) and subcutaneous regions (right image) of the injection site for vehicle (A, B), Cv-PC with 10% LD_{50} (C, D), Cv-PC with 25% LD_{50} (E, F), and Cv-PC with 50% LD_{50} (G, H) in naïve rats. The needle insertion site is marked with a black dot on the epidermis. No distinguishable inflammation is observed in either the vehicle preconditioned or the 10% LD_{50} Cv-PC. Local inflammation is caused by the 25% and 50% LD_{50} of *C. helleri* venom. 25% LD_{50} causes swelling (black arrowhead) and redness, while 50% LD_{50} also causes significant hemorrhage

(white arrowhead). Additionally, 50% LD_{50} also had bruising on the epidermis (orange arrowhead).

DISCUSSION

Brain edema is a serious complication of neurosurgery. Given the fixed space within the cranium, postoperative brain edema can lead to devastating complications such as brain herniation (Bruder and Ravussin 1999). This study presents a novel preconditioning method to reduce brain edema by prompting endogenous protective mechanisms prior to injury.

Our hypothesis was that Cv-PC would attenuate the increase in brain water content by SBI in rats. Cv-PC with 10% of the LD_{50} with *C. helleri* venom reduces brain water content by approximately 1.2% and improves neurological function 24 and 72 h after SBI. Our SBI model is characterized by about a 3% increase in brain water content compared to sham rats (data presented within, Matchett et al. 2006; Yamaguchi et al. 2007), which translates into a 23% increase in tissue volume (or swelling) due to SBI (Marmarou et al. 2000; Keep et al. 2012). However, in SBI animals receiving Cv-PC, the brain water content was increased by 1.7% compared to sham, which translates into a 9% increase in brain tissue swelling (Keep et al. 2010). So, it follows that although the brain water content was only reduced by 1.2% from Cv-PC, the amount of brain swelling was decreased from 23% (for SBI+vehicle) to only 9% (for SBI+Cv-PC) which is equivalent to approximately a 60% reduction in brain swelling following venom preconditioning.

Herein, we found that Cv-PC with *C. helleri* venom attenuates COX-2 expression in the brain, as well as PGE2 expression in the brain. Moreover, inhibition of COX-2 preceding venom injection prevents the reduced COX-2 expression. This suggests that Cv-PC induces tolerance of the brain to the COX-2 conversion of arachidonic acid into PGE2, thereby leading to reduced COX-2 and PGE2 in the brain 24 h after SBI in rats (Figure 6). Previous reports have shown that attenuating brain COX-2 expression can reduce SBI-induced brain water content increases, and that inhibiting COX-2 prior to a preconditioning stimulus leads to reversal of the beneficial effects (i.e. brain water content, COX-2 expression, and functional outcome) (Jadhav et al. 2009).

PLA2 is a large component of numerous snake venoms (Mackessy 1988), including *C. helleri* venom (Gren et al. 2017; Mackessy 1988). PLA2 is documented to be a potent neurotoxin in snake venoms (Gren et al 2017; Sunagar et al. 2014), leading to local and systemic inflammation, including

swelling (Teixeira et al. 2003). The activation of inflammation from snake venoms have shown to primarily include release of arachidonic acid caused by PLA2 enzymatic activity (Teixeira et al. 2003), thereby activating downstream enzymes, such as COX-2, leading to the release of inflammatory factors, such as PGE2. Herein, our data supports the findings of others that PLA2 in snake venom leads to activation of inflammation via the arachidonic acid/COX-2/PGE2 pathway. We extended the field of knowledge on this subject by showing that preconditioning with snake venom containing PLA2 can lead to sub-injury threshold activation of the arachidonic acid/COX-2/PGE2 pathway, thereby conferring protection against SBI-induced neuroinflammation.

Study Limitations and Future Studies

Our study is not without limitations. Indeed, one limitation of this study is that crude venom from *C. helleri* was used. Snake venoms contain a myriad of protein and non-protein components, leading to numerous physiological and off-target effects. Purification and characterization of proteins in the venom of *C. helleri* needs to be explored in future studies to increase the clinical feasibility of Cv-PC. This will also enable us to determine the protein(s) responsible for Cv-PC via the COX-2 pathway, as well as explore other pathways which might be involved in Cv-PC.

Venoms are well-documented to have both beneficial mechanisms and adverse effects. One adverse effect which needs to be examined in greater detail is the extent to which *C. helleri* venom induces local (at the site of administration), systemic, and neuro-inflammation. In future studies, we will measure the inflammatory response of the local, systemic, and central nervous systems towards single, as well as multiple, sub-lethal *C. helleri* venoms to determine these adverse effects.

Another limitation of this study is the use of normal saline as the control. While normal saline was used as the vehicle of the crude venom, a better control would have been the crude venom without PLA2 (the molecule proposed to induce the observed benefits). However, this control will be used in our future studies when we begin testing the fractionated *C. helleri* venom.

A final caveat of this study is the LD_{50} value for *C. helleri*. The LD50 value for *C. helleri* venom has been shown to have large variability, in part due to geographic location (Gren et al. 2017). The LD50

value for subcutaneous injection of *C. helleri* venom used within was based on the work of Minton (Minton 1956). Therefore, care needs to be taken when using the LD_{50} values mentioned within this manuscript.

Acknowledgements

This work was supported by NIH grant R01NS084921 to J.H. Zhang.

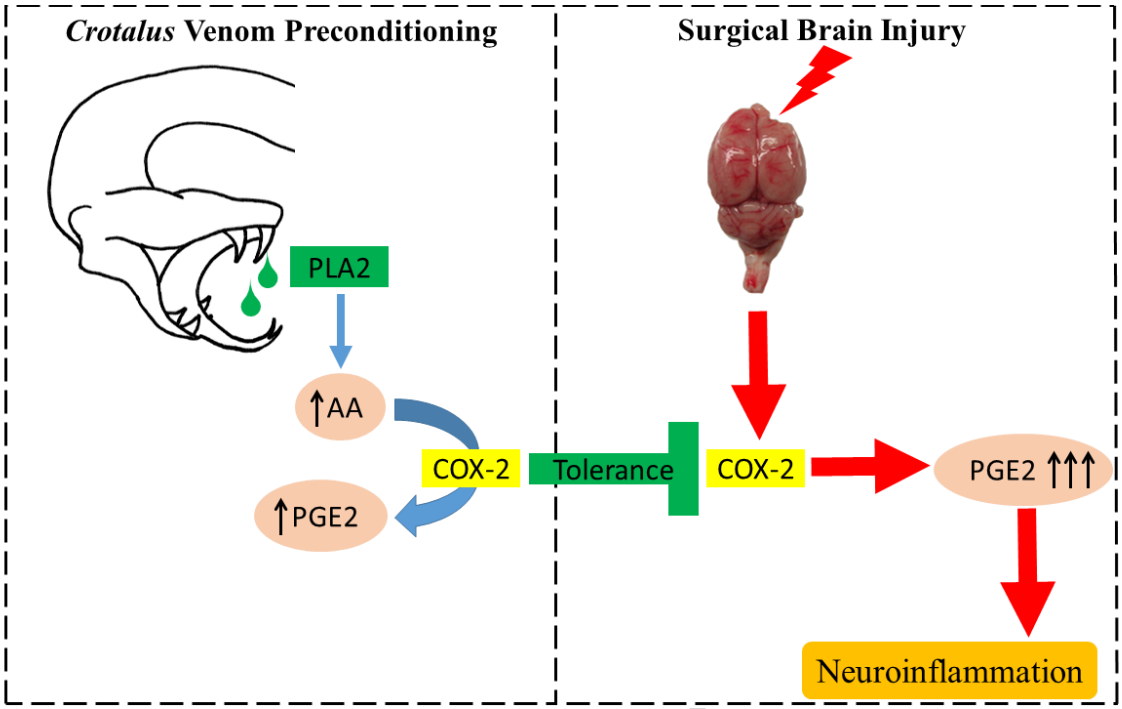

Figure 6. Schematic of preconditioning by *C. helleri* to reduce COX-2 and its downstream product, PGE2. Administering *C. helleri* venom in sublethal doses (i.e. 10% LD_{50}) leads to COX-2 activation and minor production of PGE2 from arachidonic acid (which is induced by snake venom PLA2). In small amounts, this COX-2 activation induces the brain to develop a tolerance towards COX-2 activation and downstream signaling. Thus, when an injurious event occurs (i.e. SBI), the brain is able to cope with the injury cascade much better, in part by reduced COX-2 expression.

References

Bruder, N. and P. Ravussin (1999). "Recovery from anesthesia and postoperative extubation of neurosurgical patients: a review." J Neurosurg Anesthesiol **11**(4): 282-293.

Bush, S. P. and E. Siedenburg (200). "Neurotoxicity associated with suspected southern Pacific rattlesnake (Crotalus viridis helleri) envenomation." Wilderness Environ Med **10**:247-249.

Decq, P., C. Le Guerinel, P. Brugieres, M. Djindjian, D. Silva, Y. Keravel, E. Melon and J. P. Nguyen (1998). "Endoscopic management of colloid cysts." Neurosurgery **42**(6): 1288-1294; discussion 1294-1286.

French, W. J., W. K. Hayes, S. P. Bush, M. D. Cardwell, J. O. Bader, and E. D. Rael (2004). "Mojave toxin in venom of Crotalus helleri (Southern Pacific Rattlesnake): molecular and geographic characterization." Toxicon **44**:781-791.

Garcia, J. H., S. Wagner, K. F. Liu and X. J. Hu (1995). "Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation." Stroke **26**(4): 627-634; discussion 635.

Gerzeny, M. and A. R. Cohen (1998). "Advances in endoscopic neurosurgery." AORN J **67**(5): 957-961, 963-955.

Gren, E. C. K., W. Kelln, C. Person, J. G. McCabe, R. Kornhauser, A. J. Hart, K. Erbas-White, L. R. Pompe, and W. K. Hayes (2017). "Geographic variation of venom composition and neurotoxicity in the rattlesnakes *Crotalus ogreganus* and *C. helleri*: Assessing the potential roles of selection and neutral evolutionary processes in shaping venom variation." Pages 228-252. In: The Biology of Rattlesnakes II. Editors: MJ Dreslik, WK Hayes, SJ Beaupre, and SP Mackessy. Rodeo, NM: ECO Herpetological Publishing and Distribution.

Hellwig, D., H. Bertalanffy, B. L. Bauer and W. Tirakotai (2003). "Pontine hemorrhage." J Neurosurg **99**(4): 796; author reply 796-797.

Jadhav, V., G. Matchett, F. P. Hsu and J. H. Zhang (2007a). "Inhibition of Src tyrosine kinase and effect on outcomes in a new in vivo model of surgically induced brain injury." J Neurosurg **106**(4): 680-686.

Jadhav, V., R. P. Ostrowski, W. Tong, B. Matus, R. Jesunathadas and J. H. Zhang (2009). "Cyclo-oxygenase-2 mediates hyperbaric oxygen preconditioning-induced neuroprotection in the mouse model of surgical brain injury." Stroke **40**(9): 3139-3142.

Jadhav, V., I. Solaroglu, A. Obenaus and J. H. Zhang (2007b). "Neuroprotection against surgically induced brain injury." Surg Neurol **67**(1): 15-20; discussion 20.

Kapinya, K. J., D. Lowl, C. Futterer, M. Maurer, K. F. Waschke, N. K. Isaev and U. Dirnagl (2002). "Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent." Stroke **33**(7): 1889-1898.

Keep, R. F., Y. Hua, and G. Xi (2012). "Brain water content. A misunderstood measurement?" Transl Stroke Res **3**:263-265.

Mackessy, S.P. (1988) "Venom ontogeny in the pacific rattlesnakes Crotalus veridis helleri and C. V. oreganus," Copeia **1988**(1):92-101.

Marmarou, A., P. P. Fatouros, P. Barzo, G. Portella, M. Yoshihara, O. Tsuji, T. Yamamoto, F. Laine, S. Signoretti, J. D. Ward, M. R. Bullock, and H. F. Young (2000) "Contribution of edema and cerebral blood volume to traumatic brain swelling in head-injured patients." J Neurosurg **93**:183-193.2000

Matchett, G., J. Hahn, A. Obenaus and J. Zhang (2006). "Surgically induced brain injury in rats: the effect of erythropoietin." J Neurosci Methods **158**(2): 234-241.

Minton, S. A. (1956). "Some Properties of North American Pit Viper Venoms and Their Correlation with Phylogeny." Pages: 145-151. In Venoms. Editors: EE Buckley and N Porges. Washington D.C.: American Association for the Advancement of Science.

McBride, D. W., Y. Wang, P. Sherchan, J. Tang, and J. H. Zhang (2015). "Correlation between subacute sensorimotor deficits and brain water content after surgical brain injury in rats." Behav Brain Res **1**(290):161-171.

Sherchan. P., L. Huang, Y. Wang, O. Akyol, J. Tang, and J. H. Zhang (2016). "Recombinant Slit2 attenuates neuroinflammation after surgical brain injury by inhibiting peripheral immune cell infiltration via Robo1-srGAP1 pathway in a rat model." Neurobiol Dis **85**:164-173.

Sunagar, K., E. A. B. Undheim, H. Scheib, E. C. K. Gren, C. Cochran, C. E. Person, I. Koludarov, W. Kelln, W. K. Hayes, G. F. King, A. Antunes, and B. G. Fry (2014). "Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): Biodiscovery, clinical and evolutionary implications." J Proteomics **99**:68-83.

Tang, J., J. Liu, C. Zhou, J. S. Alexander, A. Nanda, D. N. Granger and J. H. Zhang (2004). "Mmp-9 deficiency enhances collagenase-induced intracerebral hemorrhage and brain injury in mutant mice." J Cereb Blood Flow Metab **24**(10): 1133-1145.

Teixeira, C. F., E. C. Landucci, E. Antunes, M. Chacur, and Y. Cury (2003). "Inflammatory effects of snake venom myotoxic phospholipases A2." Toxicon **42**(8):947-962.

Wada, K., M. Ito, T. Miyazawa, H. Katoh, H. Nawashiro, K. Shima and H. Chigasaki (1996). "Repeated hyperbaric oxygen induces ischemic tolerance in gerbil hippocampus." Brain Res **740**(1-2): 15-20.

Yamaguchi, M., V. Jadhav, A. Obenaus, A. Colohan, and J. H. Zhang (2007). "Matrix metalloproteinase inhibition attenuates brain edema in an in vivo model of surgically-induced brain injury." Neurosurgery **61**:1067-1075; discussion 1075-1066.

Highlights

- Inflammatory mediators compromise blood-brain barrier integrity after surgical brain injury.
- *C. helleri* venom preconditioning is proposed to reduce postoperative brain edema in rats.
- The mechanism relies on downregulation of the PLA_2/COX-2 inflammatory pathway.