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Crotalus Snake Venom Preconditioning to Prevent Surgical Brain Injury

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Crotalus Snake Venom Preconditioning to Prevent Surgical Brain Injury

by

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ABBREVIATIONS

SBI	Surgical brain injury
SVMP	Snake venom metalloproteinase
PLA_2	Phospholipase A2
COX-2	Cyclooxygenase-2
VEGF	Vascular endothelial growth factor
ZO-1	Zona occludins-1
BBB	Blood-brain barrier
MMP	Matrix metalloproteinase
HBO	Hyperbaric oxygen
HBO-PC	Hyperbaric oxygen preconditioning
ATA	Absolute atmosphere
ROS	Reactive Oxygen Species
HIF-1	Hypoxia-inducible factor-1a
iNOS	Inducible NO synthase
KATP	ATP-sensitive potassium channel
CNS	Central nervous system
NMDA	N-methyl-D-aspartate
Cv	*Crotalus* venom
PC	Preconditioning
Cv-PC	*Crotalus* venom preconditioning
PGE_2	Prostaglandin E_2

ABSTRACT OF THE DISSERTATION

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by

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Preventive measures are increasingly relevant to medical practice. Preconditioning, a preemptive therapy that administers mildly harmful stimuli to induce endogenous protective mechanisms before major injury, has been shown to minimize injury in many animal models. Given the elective nature of most neurosurgical procedures, the surgical brain injury (SBI) rodent model provides an ideal platform for preconditioning. Our work shows that preconditioning with *Crotalus* rattlesnake venom, known for its hemorrhagic and inflammatory effects, mitigates some harmful effects of SBI. We have identified two proteins of interest in *Crotalus* venom: snake venom metalloproteinase (SVMP), an enzyme with hemorrhagic effects, and phospholipase A2 (PLA_2), an enzyme upstream to cyclooxygenase-2 (COX-2) in the inflammatory cascade. We have found that preconditioning *Crotalus* venom increases endogenous fibrinogen, decreases perioperative hemorrhage, attenuates COX-2 activity, and reduces postoperative brain edema. Further understanding of these enzymes may yield a novel, preventive approach to reducing perioperative hemorrhage and edema in elective neurosurgical procedures.

CHAPTER ONE

INTRODUCTION TO SURGICAL BRAIN INJURY AND NEUROPROTECTIVE PRECONDITIONING THERAPIES

Adapted from

Kim, C. H., Chen H., Zhang, J. H. (2013). Preconditioning for surgical brain injury. In *Innate Tolerance in the CNS: Translational Neuroprotection by pro- and Post-Conditioning* *(485-498). New York, NY: Springer Science.

Surgical Brain Injury

The delicate and complex architecture of the brain presents significant challenges for neurosurgery. Beneath the skull and meninges, the brain is highly susceptible to mechanical injury. Incision, retraction, and electrocauterization—all necessary neurosurgical maneuvers—can cause collateral damage to healthy tissue at the periphery of the operative site. Brain edema and hemorrhage are serious complications that commonly develop following neurosurgical procedures and may lead to further injury from hypoperfusion or cell death (Bruder and Ravussin 1999). Currently, surgical brain injury (SBI) is not specifically treated but is rather left to heal on its own.

The complications of SBI may also hinder therapeutic approach. The current medical-legal climate has led to defensive medical practice by physicians in high-risk specialties. In a study published in the Journal of the American Medical Association, nearly 75% of polled neurosurgeons admitted to avoiding certain procedures or high-risk patients (Studdert, Mello et al. 2005). Diminishing perioperative risks could expand the possibility of more aggressive surgical interventions. In addition, with the rising costs of medical care, any measures to simplify perioperative care would be invaluable. Even neurosurgical patients without life-threatening complications must be monitored closely in the critical care unit. Thus, the benefits of limiting complications of SBI extend beyond improving morbidity and mortality.

While preconditioning strategies have demonstrated promising neuroprotective effects for stroke and traumatic brain injury, they are of limited

clinical use because these injuries are not anticipated. In contrast, the predictable nature of SBI offers the opportunity to establish preemptive measures. To date, developing less invasive surgical methods has been the predominant strategy for limiting SBI, with relatively little research focused on understanding its biochemical pathophysiology. Thus, therapeutic strategies for SBI have yet to be adequately explored. In this chapter, we will describe SBI's animal model, review its pathophysiology, and discuss potential preconditioning therapies for SBI.

Surgical Brain Injury Rodent Model

Despite its widespread impact and predictability, SBI is clinically addressed by rather nonspecific postoperative care. In order to facilitate the study of specific treatments for SBI, the *in vivo* model for SBI was recently developed by John H. Zhang and colleagues (Matchett, Hahn et al. 2006, Jadhav, Solaroglu et al. 2007, Yamaguchi, Jadhav et al. 2007). This rodent model was designed to mimic surgically induced brain injury. In brief, the rodent brain is exposed in a small window in the cranium through which tissue resection is performed. The margins of resection are delineated by distance from the bregma, as shown in Fig. 1.1. This partial right front lobectomy simulates the injuries sustained by neural tissue from standard neurosurgical maneuvers. The model provides for a consistently measureable edema by brain water content in perilesional tissue and neurological deficits following SBI. The model mimics injuries characteristic of general neurosurgical procedures, producing reproducible brain tissue loss and injury that is representative of routine

neurosurgeries in clinical practice and provides a platform from which to study the molecular mechanisms involved in SBI pathophysiology as well as potential therapeutic agent.

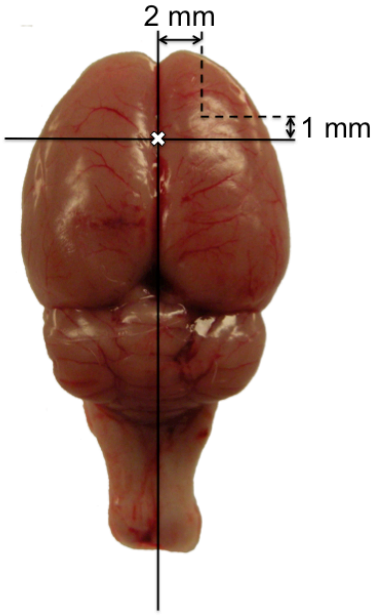

Figure 1.1 Partial right frontal lobectomy. Two incisions (interrupted lines) are made leading away from the bregma (white ×) along the sagittal and coronal planes 2 mm lateral and 1 mm proximal to the sagittal and coronal sutures, respectively.

Pathophysiology of Surgical Brain Injury

Surgical brain injury is caused by both primary and secondary injury mechanisms. Primary injury, inflicted directly by mechanical forces at the time of injury, is largely inescapable and irreversible. Secondary injury entails a cascade of cellular and metabolic disruption triggered by the primary insult. Typical biochemical disturbances of secondary injury involve the generation of toxic and inflammatory molecules, such as reactive oxygen species, prostaglandins, and cytokines. The principal complications that result from such mechanisms after SBI are brain edema, cell death, and hemorrhage.

Brain Edema

Localized brain edema has been shown to be a major postoperative complication of SBI. Many rodent studies indicate that the brain water content of tissue surrounding the resection site is increased by approximately 3% during the first three postoperative days and gradually resolves by a week after surgery (Matchett, Hahn et al. 2006, Yamaguchi, Jadhav et al. 2007). This sequence was further supported with measured apparent diffusion coefficients by magnetic resonance imaging (Matchett, Hahn et al. 2006). Brain edema is believed to develop from both vasogenic and cytotoxic mechanisms. In vasogenic edema formation, loss of blood-brain barrier (BBB) integrity causes extravasation of proteins from the vascular compartment and subsequent fluid accumulation in brain tissue. Cytotoxic edema is formed by fluid accumulation in cells from

improper ion balance. Brain edema is followed by increased intracranial pressure, which may lead to local ischemia, herniation, and cell death.

SBI leads to abnormal extravasation of proteins from the vasculature, suggesting loss of BBB integrity (Matchett, Hahn et al. 2006, Jadhav, Solaroglu et al. 2007, Bravo, Matchett et al. 2008, Di, Yan-Ting et al. 2008, Jadhav, Yamaguchi et al. 2008, Lee, Jadhav et al. 2008, Hao, Wu et al. 2009). While a clear mechanism for SBI-induced BBB disruption has yet to emerge, growth factors and inflammatory pathways have been implicated in previous studies. SBI is characterized by increased expression of vascular endothelial growth factor (VEGF) and decreased expression of zona occludens-1 (ZO-1), a tight junction protein; inhibition of ERK1/2 phosphorylation suppresses VEGF expression and salvages ZO-1 expression (Jadhav, Matchett et al. 2007). Another growth factor, erythropoietin, exacerbates brain edema in SBI (Matchett, Hahn et al. 2006). Other studies have shown increased extracellular matrix degradation by matrix metalloproteinases (MMPs) in SBI, compromising the basement membrane of endothelial cells (Yamaguchi, Jadhav et al. 2007). A key enzyme in the production of prostaglandins and other inflammatory mediators, cyclooxygenase-2 (COX-2) appears to play a role in the induction of brain edema in SBI as well (Jadhav, Ostrowski et al. 2009, Jadhav, Ostrowski et al. 2010). Lipid peroxidation by reactive oxygen species (ROS) has been implicated in BBB disruption (Lo, Bravo et al. 2007, Lee, Jadhav et al. 2008, Lee, Jadhav et al. 2009). Inflammatory mediators are notorious participants in BBB regulation, yet they alone do not dictate the development of edema following SBI. Hyong et al.

demonstrated the capacity of rosiglitazone to decrease inflammatory markers, such as tumor necrosis factor-a, interleukin-b, and myeloperoxidase, following SBI; however, brain edema and BBB disruption persisted (Hyong, Jadhav et al. 2008).

Cell Death

Apoptosis and other forms of cell death have also been implicated in SBI. Several studies have reported apoptotic changes at the perilesional site in SBI (Matchett et al. 2006; Bravo et al. 2008; Sulejczak et al. 2008). Matchett et al. demonstrated apoptotic neuronal death by positive triple immunostaining for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, cleaved caspase 3, and NeuN (Matchett, Hahn et al. 2006). Neuronal apoptosis was later shown to be accompanied by astrogliosis in the perilesional area (Sulejczak, Grieb et al. 2008).

Perioperative Hemorrhage

Hemorrhage is a major obstacle in neurosurgery and likely contributor to SBI. Postoperative hemorrhage in SBI may trigger injury by disrupting the BBB by similar mechanisms as observed in the intracerebral hemorrhage and subarachnoid hemorrhage stroke models. Intraoperative bleeding can obscure the surgical field and cause local ischemic insult, as well as systemic cardiovascular and respiratory instability. Extensive losses may require blood transfusions, prolonging the surgery. Procedure duration, extended by efforts to

stem bleeding, is itself a risk. Achieving hemostasis is unquestionably critical in all types of surgeries but poses unique challenges in neurosurgery. Many surgical methods for controlling bleeding, such as clamping or suturing, are unacceptable in neurosurgery because of the delicate nature of brain tissue. Since Harvey Cushing first introduced its surgical use in 1928, electrocauterization has been the primary neurosurgical method of reducing intraoperative bleeding (Voorhees, Cohen-Gadol et al. 2005). While a groundbreaking addition to neurosurgery, electrocauterization itself can cause the destruction of healthy brain tissue either directly or by thermal injury.

Neuroprotective Preconditioning Therapies

Currently, clinical management of surgical brain injury is limited to nonspecific postoperative care. Many promising therapeutic agents and strategies to mitigate complications of SBI have been observed in animal models (summarized in Table 1.1); however, nearly all of these studies utilized pre- or post-treatments. SBI presents a unique opportunity to test neuroprotection by preconditioning therapies that may prove clinically relevant. In the following text, we propose the use of hyperbaric oxygen, inhalatory anesthetic, and N-methyl-D-aspartate as preconditioning agents for SBI.

Table 1.1. *Experimental animal studies of therapeutic agents for surgical brain injury.*

Name, Year Journal	Animal Model		Treatment	SBI Findings, Treatment Outcome
Matchett, 2006 ***J Neurosci Methods***	Rat (male)	Erythropoietin pretreatment	4 daily doses (5000U/kg i.p.) Starting 1 day prior to surgery	SBI: ↑ brain water content (BWC) Treatment (Tx): Harmful, ↑↑ BWC
Lo, 2007 ***Neurosci Lett***	Mouse (male)	NADPH oxidase KO or apocynin pretreatment	1 dose apocynin (5 mg/kg i.p.) 30 min before surgery	SBI: ↑ BWC, ↓neurological score (NS) KO: ↑neuroscore Tx: No effect
Jadhav, 2007 ***J Neurosurg***	Rat (male)	PP1 pretreatment	1 dose (1.5 mg/kg i.p.) 45 min before surgery	SBI: ↑VEGF, ↑p-ERK1/2, ↓ZO-1, ↑BWC Treatment: ↓VEGF, ↓p-ERK1/2, ↑ZO-1, ↓BWC
Yamaguchi, 2007 ***Neurosurg***	Rat (male)	MMP inhibitor-1 pretreatment	3 daily doses, starting 2 days before surgery 1 dose, 60 min before surgery	SBI findings: ↑BWC, ↓NS Tx: ↓BWC
Lee, 2008 ***Acta Neurochir Suppl***	Rat (male)	Simvastin pretreatment	7 daily doses (i.p.) Starting 6 days prior to surgery	SBI: ↑BWC, ↓neurological score Tx: No effect
Lee, 2008 ***Acta Neurochir Suppl***	Rat (male)	Melatonin pretreatment	1 dose (5, 15, or 15 mg/kg i.p) 1 hr before surgery	SBI: ↑BWC, ↓NS, ↑lipid peroxidation (LPO) Tx (5, 15 mg/kg dose): ↓BWC, ↑NS, ↓LPO Tx (150 mg/kg dose): ↑↑BWC, ↓↓NS, ↑↑LPO
Bravo, 2008 ***Brain Res***	Mouse (male)	L-histidine and thioperamide posttreatment	1 dose (L-histidine 1000 mg/kg i.p, thioperamide 5 mg/kg i.p) Immediately following surgery	SBI: ↑BWC, ↓NS Tx: ↑↑BWC
Hyong, 2008 ***Brain Res***	Rat (male)	Rosiglitazone pretreatment	3 doses (1 or 6 mg/kg i.p.) 30 min before, 30 min after, and 4 hr after surgery	SBI: ↑BWC, ↓NS, ↑myeloperoxidase activity (MPO), ↑TNF-α, ↑IL-1β Tx : ↓MPO, ↓TNF-α, ↓IL-1β
Di, 2008 ***Neurosci Lett***	Rat (male)	Aminoguanidine posttreatment	1 dose (75, 150, or 300 mg/kg i.p) Immediately following surgery	SBI: ↑BWC, ↓NS, ↑TNF-α, ↑NF-κB Tx (150 mg/kg): ↓BWC, ↑NS, ↓TNF-α, ↓NF-κB Other concentrations of drug showed no effect
Hao, 2009 ***Brain Res***	Rat (male)	Aminoguanidine posttreatment	1 dose (75, 150, or 300 mg/kg i.p) Immediately following surgery	SBI: ↑malondialdehyde (MDA), ↓glutathione (GSH), ↑aquaporin-4 (AQ-4) Tx (150 mg/kg): ↓MDA, ↑GSH, ↓AQ-4
Jadhav, 2009 ***Stroke***	Mouse (male)	Hyperbaric oxygen preconditioning	5 daily doses (1 hr O_2 2.5 ATA) Starting 5 days prior to surgery	SBI: ↑BWC, ↓NS, ↑cyclooxgenase-2 (COX-2), ↑hypoxia-inducible factor-1α (HIF-1α) Tx: ↓BWC, ↑NS, ↓COX-2, ↓HIF-1 α

Hyperbaric Oxygen

To date, hyperbaric oxygen (HBO) is the only preconditioning therapy that has been successful in the surgical brain injury model. Jadhav et al. investigated brain edema and neurological outcome with HBO preconditioning (HBO-PC) treatment in mice subjected to SBI (Jadhav, Ostrowski et al. 2009, Jadhav, Ostrowski et al. 2010). The mice were treated with 100% for 1 h at 2.5 absolute atmosphere (ATA) for five consecutive days prior to surgery. Examined at 24 h and 72 h after surgery, the HBO-treated mice subjected to SBI showed both decreased brain water content and improved neurological status, which was assessed by 21-point sensorimotor scoring and wire-hang and beam-balance tests. The authors suggested that the benefits of HBO-PC for SBI are mediated by COX-2, as its inhibition reversed the effects, and SBI-induced COX-2 overexpression was attenuated by HBO-PC.

HBO preconditioning has demonstrated neuroprotective effects in several models of stroke and brain injury; however, its mechanism is not fully understood. Most studies have shown that the key benefit of HBO-PC is apoptosis inhibition. Ostrowski et al. suggested that HBO-PC reduced the activation of the p38 apoptotic pathway and upregulated the expression of neurotrophins, such as brain-derived neurotrophic factor (Ostrowski, Graupner et al. 2008). Hypoxia-inducible factor-1alpha (HIF-1) has also been implicated as a mediator of HBO-PC. A transcription factor expressed in response to hypoxia, HIF-1 governs genes responsible for adaptive responses (Carmeliet, Dor et al. 1998); however, the actions of HIF-1 are complex, as excessive accumulation of

HIF-1 results in the activation of proapoptotic and proinflammatory pathways (Halterman, Miller et al. 1999, Li, Zhou et al. 2005). Jadhav et al. observed increased HIF-1a expression following SBI that was reduced by 5 days of HBO-PC at 2.5 ATA (Jadhav, Ostrowski et al. 2009). In contrast, Peng et al. demonstrated that the same HBO-PC treatment upregulated HIF-1a expression in a mouse hypoxia model (Peng, Ren et al. 2008). In short, HIF-1a needs more experimental study to clarify its role in HBO-PC.

HBO-PC has also been shown to activate endogenous antioxidants. HBO appears to present an oxidative challenge to which the brain upregulates the activity of endogenous antioxidants, such as superoxide dismutase, in global ischemia models (Wada, Miyazawa et al. 2001, Nie, Xiong et al. 2006). The induction of antioxidant systems prior to injury would bolster the ability to limit oxidative damage and cell membrane disruption by ROS following brain insult, preserving BBB integrity (Fig. 1.2).

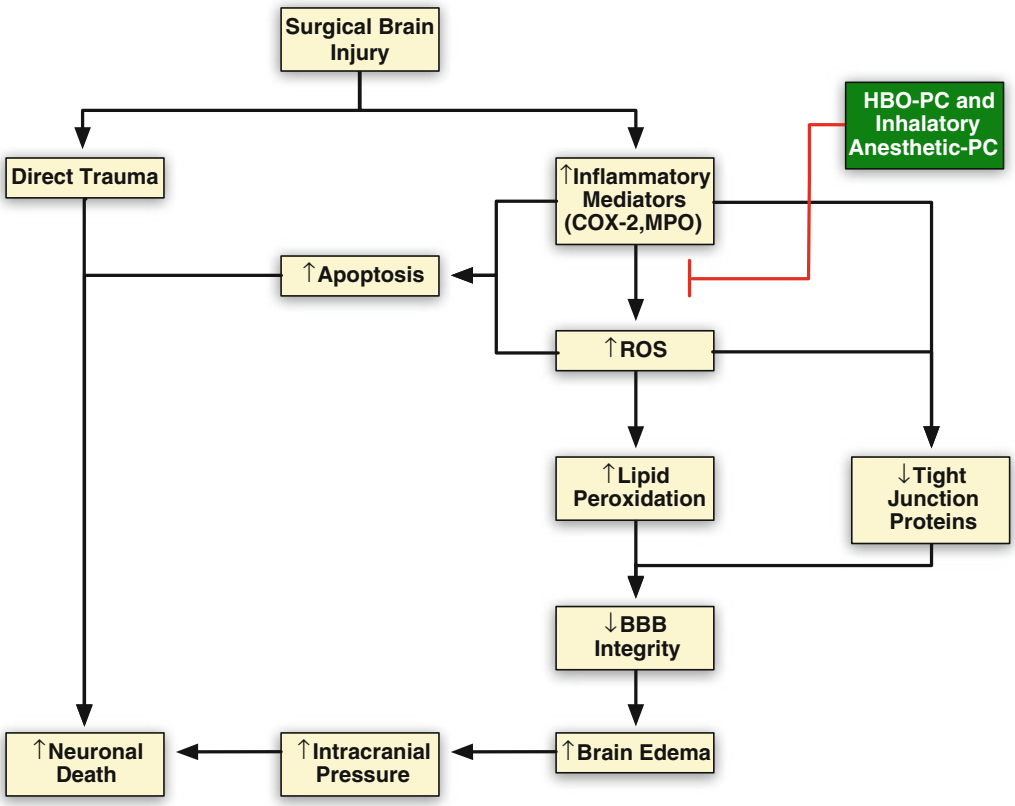

Figure 1.2 Proposed scheme of surgical brain injury pathophysiology and potential preconditioning interventions. *HBO* hyperbaric oxygen, *PC* preconditioning, *COX-2* cyclooxygenase-2, *MPO* myeloperoxidase, *ROS* reactive oxygen species.

Historically, the clinical use of HBO-PC has been controversial because of oxygen toxicity issues. HBO generated oxidative stress on vulnerable tissues, such as the lung, is a valid concern; however, the hazards of HBO may be somewhat overstated. Research studies and clinical trials have quelled fears of toxicity and established that HBO treatment pressure at 1.5 ATA is well tolerated without permanent pulmonary complications (Holbach, Schroder et al. 1972, Holbach, Caroli et al. 1977, Rockswold, Ford et al. 1992, Rockswold, Rockswold et al. 2007). The variability in HBO-PC dosing regimen presents another challenge; studies have differed in exposure pressure, timing, number, and duration of sessions. This complicates the translation of HBO-PC into the clinic. Thus, the challenges of introducing HBO-PC as an acceptable therapy for SBI are to establish efficacy and safety of treatment.

Inhalatory Anesthetics

Preconditioning with inhalatory anesthetics has shown some promising neuroprotective results in experimental stroke models—studies have shown reduction of infarct volumes and improvement of neurological deficit scores in cerebral ischemia (Kapinya, Lowl et al. 2002, Zhao and Zuo 2004, Zheng and Zuo 2004, Payne, Akca et al. 2005, Liu, Xiong et al. 2006). Some studies have suggested the induction of nitric oxide synthase as a potential mechanism for inhalatory anesthetic preconditioning. Kapinya et al. demonstrated the increase of inducible NO synthase (iNOS) from inhalatory anesthetic preconditioning in an ischemic model; the reduction of infarct was eliminated with the administration of

an iNOS inhibitor (Kapinya, Lowl et al. 2002). Another study by Zhao and Zuo showed analogous results in a neonatal hypoxia-ischemia model (Zhao and Zuo 2004). KATP channels in the brain, especially those of mitochondrial origin, appear to play a major role in reducing neuronal death from brain injury. Preconditioning with inhalatory anesthetics is thought to be neuroprotective through KATP channel opening and activation; the inhibition of KATP channels has been shown to abolish the beneficial effects of inhalatory anesthetic preconditioning in both *in vitro* and *in vivo* studies (Xiong, Zheng et al. 2003, Kehl, Payne et al. 2004, Kaneko, Yokoyama et al. 2005). Adenosine A1 receptor activation has been suggested to be a trigger for KATP channel opening in inhalatory anesthetic preconditioning (Liu, Xiong et al. 2006); however, downstream effects of KATP channel opening that mediate neuroprotection are not well understood. Studies investigating the action of inhalatory anesthetic preconditioning of cardiac tissue have demonstrated improved mitochondrial energy generation and increased ROS production that was dependent on mitochondrial KATP channel opening (Tanaka, Weihrauch et al. 2003, O'Rourke 2004) (O'Rourke 2004; Tanaka et al. 2003). Thus, KATP channel-mediated neuroprotection seen in inhalatory anesthetic preconditioning may also be mediated by ROS production, a pathway shared by other therapies, such as HBO-PC or ischemic preconditioning. More research is needed to establish downstream effects of KATP channel activation in brain.

Most preconditioning studies have utilized isoflurane because of its availability and limited systemic side effects. The few studies that have studied

the neuroprotective effects of halothane preconditioning have shown conflicting results in rodent cerebral ischemia models. Some have suggested that halothane may offer a therapeutic window of neuroprotection (Baughman, Hoffman et al. 1988, Warner, McFarlane et al. 1993, Warner, Ludwig et al. 1995, Sarraf-Yazdi, Sheng et al. 1999); however, the argument for its clinical relevance is a moot point, as halothane is no longer manufactured in the United States because of its potential hepatotoxicity (Kitano, Kirsch et al. 2007). Sevoflurane has recently been utilized for preconditioning studies and has shown encouraging results in hypoxia-ischemia models (Kehl, Payne et al. 2004, Payne, Akca et al. 2005). Xenon has also emerged as promising preconditioning agent for neuroprotection (Bantel, Maze et al. 2009, Limatola, Ward et al. 2010).

The potential induction of ROS by inhalatory anesthetic preconditioning is of particular interest for the treatment of SBI. HBO-PC has demonstrated ROS ability to activate endogenous antioxidant systems. Inhalatory anesthetics offer a potentially less hazardous and more convenient way to precondition by oxidative challenge (Fig. 1.2). Further studies are needed to examine the effects of inhalatory anesthetic preconditioning on cerebral edema and BBB integrity.

N-Methyl-D-Aspartate

Glutamate is a major excitatory neurotransmitter of the central nervous system (CNS) that is involved in the pathophysiology of brain injuries. Glutamate concentrations have been reported to rise significantly following various types of CNS injury, such as ischemia or trauma (Benveniste, Drejer et al. 1984,

Katayama, Tamura et al. 1991, Liu, Thangnipon et al. 1991). Excessive activation of ionotropic N-methyl-D-aspartate (NMDA) receptors by glutamate in the postsynaptic cell causes an influx of sodium and calcium that overwhelms the cell's ability to maintain the ion hemostasis. The accumulation of intracellular sodium may result in cellular edema, while intracellular calcium can cause the inappropriate activation of regulatory cascades that mediate cell death (Choi, Maulucci-Gedde et al. 1987, Choi 1994, Choi 1995). In addition, glutamate-induced over-activation of the CNS augments the metabolic needs of neural tissue.

In vitro studies have shown NMDA-PC to protect cultured neurons from subsequently administration of a neurotoxic concentration of glutamate (Chuang, Gao et al. 1992). Calcium involvement in NMDA-PC was confirmed by Raval and colleagues (Raval, Dave et al. 2003). Their *in vitro* study demonstrated that calcium chelation abolished the neuroprotective effects of NMDA-PC. NMDA preconditioning has been shown to reduce neuronal death from not only excitotoxic damage but from oxidative insults as well (Smith, Stone et al. 2008). Most *in vivo* NMDA-PC studies have focused on investigating its anticonvulsant effects; however, one has examined its efficacy in traumatic brain injury. In a 2010 study, Costa et al. observed that NMDA-PC improved neurological function in the mouse traumatic brain injury model (Costa, Constantino et al. 2010).

Though its clinical applicability has yet to be established, NMDA-PC has shown promising results in both *in vitro* and *in vivo* models of ischemia and CNS injury. The importance of glutamate-induced excitotoxicity in the

pathophysiological sequelae of SBI is yet unknown; however, glutamate's role in other similar insults, like traumatic brain or spinal cord injury, suggests that limiting glutamate signaling may prove beneficial in SBI as well. NMDA-PC could theoretically limit apoptosis and brain edema of SBI by a mechanism similar to that has been observed in ischemia and traumatic brain injury (Fig. 22.3). The ability of NMDA-PC to protect against neuronal death could prove clinically useful in limiting the complications of SBI.

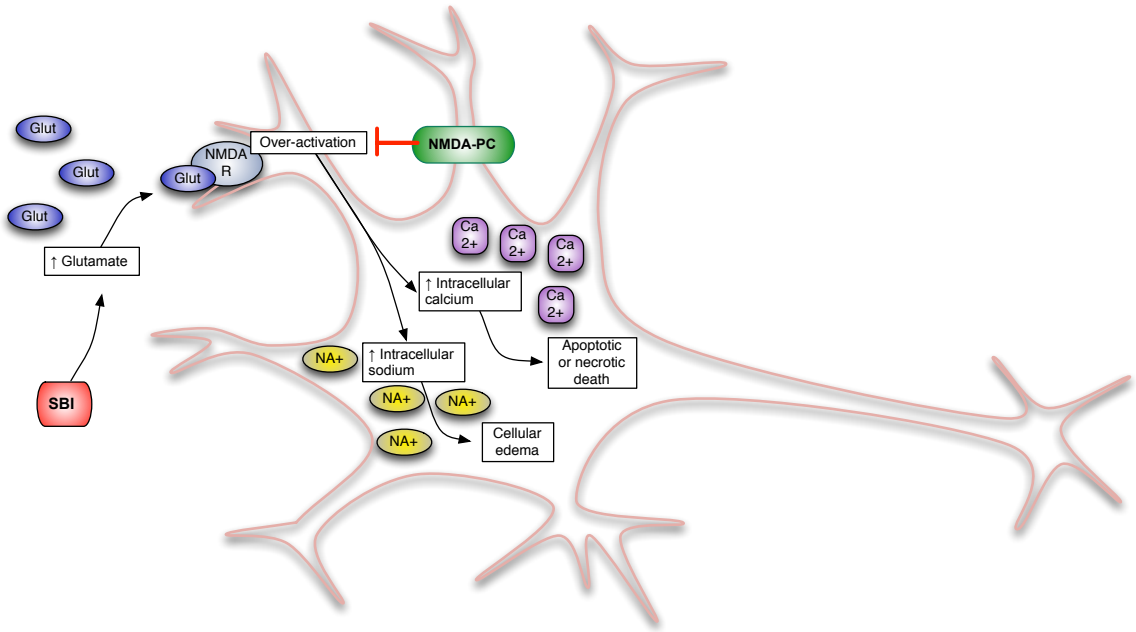

Figure 1.3. Proposed mechanism of NMDA preconditioning on surgical brain injury. NMDA preconditioning (NMDA-PC) attenuates glutamate over-activation, reducing cell edema and death. NMDA-R NMDA receptor, SBI surgical brain injury

Future Directions

The anticipatable timing of surgical brain injury provides a unique opportunity for preemptive intervention, but clinical medicine has yet to utilize preconditioning methods to protect the brain from SBI. Before these therapies can be tested by clinical trial, further *in vivo* experimental studies are needed to evaluate preconditioning agents and to provide a better mechanistic understanding of SBI pathophysiology. The pathophysiological understanding of SBI is still sparse compared that of other stroke or brain injury models. To date, SBI studies have implicated certain potential pathways, such as the p38 apoptotic and the COX-2-mediated inflammatory pathways. Further studies are needed to expand on upstream and downstream mediators of these signaling pathways in the pathogenesis of SBI.

HBO preconditioning has been shown to attenuate brain edema formation and neurological deterioration in surgical brain injury (Jadhav, Ostrowski et al. 2009, Jadhav, Ostrowski et al. 2010). While HBO-PC has demonstrated promising results in animal studies, it remains to be seen whether it is feasible therapy in the clinical setting. A hyperbaric facility may not be easily accessible to neurosurgical patients, limiting the applicability of HBO-PC for SBI. In such a case, it would be important to examine other preconditioning therapies activating similar endogenous protective mechanisms.

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CHAPTER TWO

CROTALUS SNAKE VENOM: POTENTIAL FOR PRECONDITIONING IN SURGICAL BRAIN INJURY

Therapeutic Uses of Venoms

Recent advances in science recently have piqued interest in the use of snake venom in the medical field for their effects. Snake venoms are complex concoctions of deadly and pharmacologically active proteins. Scientists from various backgrounds have studied snake venoms to understand the mechanism of actions of its toxins either to find ways to neutralize the toxin effects during envenomation or to develop uses for the toxins in both research and clinical medicine.

Snake venoms have long been studied for its effects on hemostasis. Snake venom toxins have been used to detect coagulative disorders in the medical laboratory (Marsh 2001), the anti-coagulative properties of snake venom have been studied for their potential in treating patients with hypercoagulative states, and the venoms with coagulative properties have been used to control bleeding (Earl, Masci et al. 2012). Others have been studied for potential antibacterial and anticancer properties (Nevalainen, Graham et al. 2008, Nascimento, Sancey et al. 2012, de Oliveira Junior, e Silva Cardoso et al. 2013). Only about 20% of snakes are venomous; the majority of these venom-producing snakes come from the families *Elapidae, Viperidae, Colubridae,* and *Atractaspididae* (Berling and Isbister 2015).

Crotalus Snakes and Venom

There are more than 200 species of *Viperidae*; among these are the most dangerous snakes to human beings, including the rattlesnake subfamilies *Crotalus* and *Sistrurus* (Del Brutto and Del Brutto 2012). The venoms produced by the rattlesnakes in the *Crotalus* subfamily are recognized for their inflammatory and hemorrhagic effects.

Crotalus helleri (formerly *viridis*), also known as the South Pacific rattlesnake, is a medium-sized snake that is responsible for the majority of snakebites in the coastal area of California (Wasserberger, Ordog et al. 2006, Sunagar, Undheim et al. 2014). *Crotalus atrox*, also known as the Western Diamondback rattlesnake, is the largest western rattlesnake in size in addition to being the most widely geographically spread monotypic rattlesnake species. Given its aggressive predisposition, *C. atrox* is the second highest cause of snakebite fatalities in the United States and the foremost cause in northern Mexico (Campbell and Lamar 2004).

The venom of *Crotalus* snakes consists of hemorrhagic, hemotoxic, myotoxic, and cytotoxic components, along with some amounts of neurotoxins. *Crotalus* venoms are noted to contain PLA_2 with myotoxic effects. Hemorrhagic components can lead to hemorrhage, myonecrosis, hypotension, hypovolemia, hemoconcentration, and shock (Campbell and Lamar 2004). *C. atrox* envenomation has been demonstrated to lead to persistent bleeding (Bjarnason and Tu 1978).

Crotalus Venom as a Potential Preconditioning Agent for Reducing Postoperative Inflammation in Surgical Brain Injury

As previously discussed, COX-2, an enzyme downstream of PLA_2, has been shown to mediate brain edema in SBI. COX-2 has also been identified as a potential mediator of HBO-PC in SBI—animals preconditioned for 1 hour daily for 5 days with HBO prior to the induction of SBI had significantly improved neurological function and brain edema; with HBO-PC, COX-2 levels increased by 2-fold in comparison to the 4-fold increase by the SBI, suggesting that protection conferred by HBO-PC involved increasing COX-2 to sub-injurious levels prior to SBI. Furthermore, HBO-PC's beneficial effects were reversed with the addition of selective COX-2 inhibitor to HBO-PC treatments (Jadhav, Ostrowski et al. 2009, Jadhav, Ostrowski et al. 2010).

Crotalus snake venom is known to contain PLA_2, an enzyme upstream to COX-2 in the inflammatory cascade, which converts membrane phospholipids into arachidonic acid (Bush and Siedenburg 1999, French, Hayes et al. 2004). Thus, *Crotalus* venom presents a new way to deliver sub-injurious increases in COX-2 levels in animals destined for SBI.

Crotalus Venom as a Potential Preconditioning Agent for Reducing Perioperative Hemorrhage in Surgical Brain Injury

Recent studies have highlighted low plasma fibrinogen levels as a key risk factor for perioperative hemorrhage during surgery (Gerlach, Tolle et al. 2002,

Ucar, Oc et al. 2007, Carling, Jeppsson et al. 2011, Adelmann, Klaus et al. 2014, Galas, de Almeida et al. 2014, Pillai, Fraser et al. 2014, Walden, Jeppsson et al. 2014). These studies have demonstrated that lower plasma fibrinogen, even within normal limits, are associated with increased bleeding. Fibrinogen, an acute phase reactant with a half-life of 3.74 days (Martinez, Holburn et al. 1974), serves as the substrate for fibrin clot formation and circulates at the highest concentration of all the coagulation factors (Lowe, Rumley et al. 2004). Dilutional deficiency of fibrinogen develops before the development of any other hemostatic abnormality when plasma-poor red blood cell preparations are used to replace major blood loss (Hiippala, Myllyla et al. 1995). Yet the options for increasing plasma fibrinogen are limited to transfusions of fibrinogen-containing preparations such as fresh frozen plasma, cryoprecipitate, and fibrinogen concentrate. Fresh frozen plasma (FFP) varies in fibrinogen concentration as it is collected from donors and reportedly ranges between 100-300 mg/dL (Theusinger, Baulig et al. 2011). Cryoprecipitate, obtained by concentrating FFP, has a concentration of approximately 550 mg/dL (Lee, Lee et al. 2014). Fibrinogen concentrate, manufactured from human plasma and available as a pasteurized, lyophilized powder, is available at a concentration of 2000 mg/dL (Levy, Szlam et al. 2012). A previous study estimated that in order to raise plasma fibrinogen levels from 150 mg/dL to 170 mg/dL, 14 units of FFP, 8 units of cryoprecipitate, or 2 units of fibrinogen concentrate are required (Collins, Solomon et al. 2014); thus, fibrinogen supplementation by FPP and cryoprecipitate administration is limited by the possibility of causing fluid

overload. Additionally, these transfusions are associated with complications that include allergic reaction, infections, and transfusion-related acute lung injury (Busch, Glynn et al. 2005, Inaba, Branco et al. 2010).

Crotalus venom contains SVMPs that cleave fibrinogen into fibrin split products (FSPs) in such a manner that renders it useless for fibrin clot formation (Martinez, Holburn et al. 1974, Pandya, Rubin et al. 1983). Studies have demonstrated that FSPs are capable of increasing fibrinogen plasma levels by inducing endogenous production of fibrinogen in hepatocytes (Fuller, Otto et al. 1985, Princen, Moshage et al. 1985, Amrani 1990). This distinctive degradation of fibrinogen by *Crotalus* SVMPs presents an opportunity to harness the toxic potential of the venom to elicit an endogenous response to increase plasma fibrinogen levels.

Research Questions, Hypotheses, and Specific Aims

Over 200 million major elective surgeries are performed worldwide per year (Weiser, Regenbogen et al. 2008). With the rising costs of healthcare, preventive measures grow increasingly relevant to medical practice. Having established that SBI is an ideal platform for preconditioning and *Crotalus* venom as a promising therapeutic agent, we propose that preconditioning with small doses of *Crotalus* snake venom, known for both their inflammatory and hemorrhagic effects, will lead to increase tolerance to SBI. As discussed, the inflammatory and hemorrhagic nature of *Crotalus* venoms makes them a prime candidate to expand PC therapy for preventing SBI-induced brain edema and

hemorrhage. In our studies, we hope to open a new avenue of therapy for SBI using *Crotalus* venoms by utilizing their toxic properties to preemptively upregulate the endogenous response to inflammatory and hemorrhagic injury prior to SBI onset.

Our central hypothesis is that preconditioning with *Crotalus* venom (Cv-PC), which consists of PLA_2 and SVMPs, will temper the severity of SBI by inducing innate tolerance to injury. We propose to systematically examine the effects of high and low doses and investigate the molecular mechanisms of Cv-PC by the following study aims:

Aim 1: The mechanism of edema prevention by Cv-PC is via the PLA_2/COX-2 inflammatory signaling pathway.

Our corollary hypothesis is that Cv-PC will downregulate the response of the PLA_2/COX-2 to injury, thereby reducing the inflammatory response. We will evaluate brain water content after Cv-PC, the expression of COX-2 after SBI, and evaluate the effects of antagonizing COX-2 while administering Cv-PC.

Aim 2: The mechanism of reduced hemorrhage by Cv-PC is via fibrinogen cleavage.

Our corollary hypothesis is that fibrinogen is cleaved by SVMP, increasing fibrinogen split products, leading to increased biosynthesis of plasma fibrinogen, ultimately improving clotting and reducing hemorrhage volume. We will measure hemorrhage volume, plasma fibrinogen and fibrinogen split product concentrations, determine coagulative parameters, and evaluate the effects of

antagonizing SVMP in Cv-PC. We will also fractionate the venom in order to identify the active venom components in Cv-PC.

Our long-term goal is to develop an effective, preemptive therapy for SBI that will improve patient outcome and decrease costs of perioperative care for neurosurgical patients. We hope to establish a better mechanistic understanding of SBI that will facilitate the application of preconditioning therapies in clinical practice.

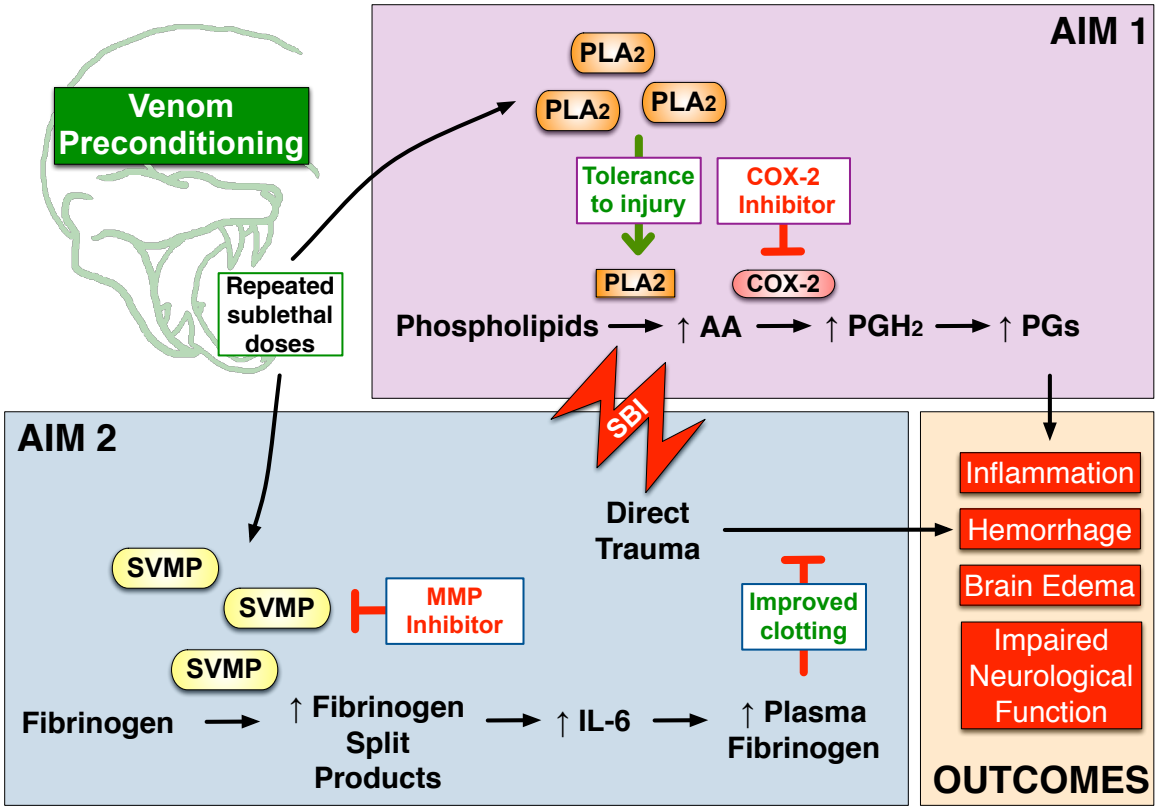

Figure 2.1. Schematic of Specific Aims. AA: arachidonic acid; COX-2: cyclooxygenase-2; IL-6: interleukin-6; MMP: matrix metalloproteinase; PG: prostaglandins; PGH_2: prostaglandin H_2; PLA_2: phospholipase 2; SVMP: snake venom metalloproteinase.

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CHAPTER THREE

CROTALUS SNAKE VENOM PRECONDITIONING REDUCES POSTOPERATIVE BRAIN EDEMA AND IMPROVES NEUROLOGICAL OUTCOMES AFTER 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, Le Guerinel et al. 1998, Gerzeny and Cohen 1998) and administering nonspecific postoperative care (Bruder and Ravussin 1999, Hellwig, Bertalanffy 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, Hahn et al. 2006, Jadhav, Matchett et al. 2007). 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, Ito et al. 1996, Kapinya, Lowl 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, Ostrowski 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.

Results

Cv-PC Reduces Brain Water Content after SBI

At 24 h after SBI, the vehicle-treated animals were observed to have increased 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%. Similarly, 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 20% LD50 had significantly reduced brain water content at both 24 h and 72 h after SBI compared to vehicle-treated animals, while Cv-PC with 10% LD50 doses showed brain water content that was lower but not statistically significant (Figure 3.1B).

Cv-PC Improves Neurological Function after SBI

To assess the neurological function after Cv-PC, the modified Garcia neurological scores were assessed (Figure 1C). 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-treated animals (18.3 ± 0.3 and 18.2 ± 0.4, respectively) scored significantly higher than vehicle-treated animals (16.4 ± 0.4 and 15.8 ± 0.6, respectively).

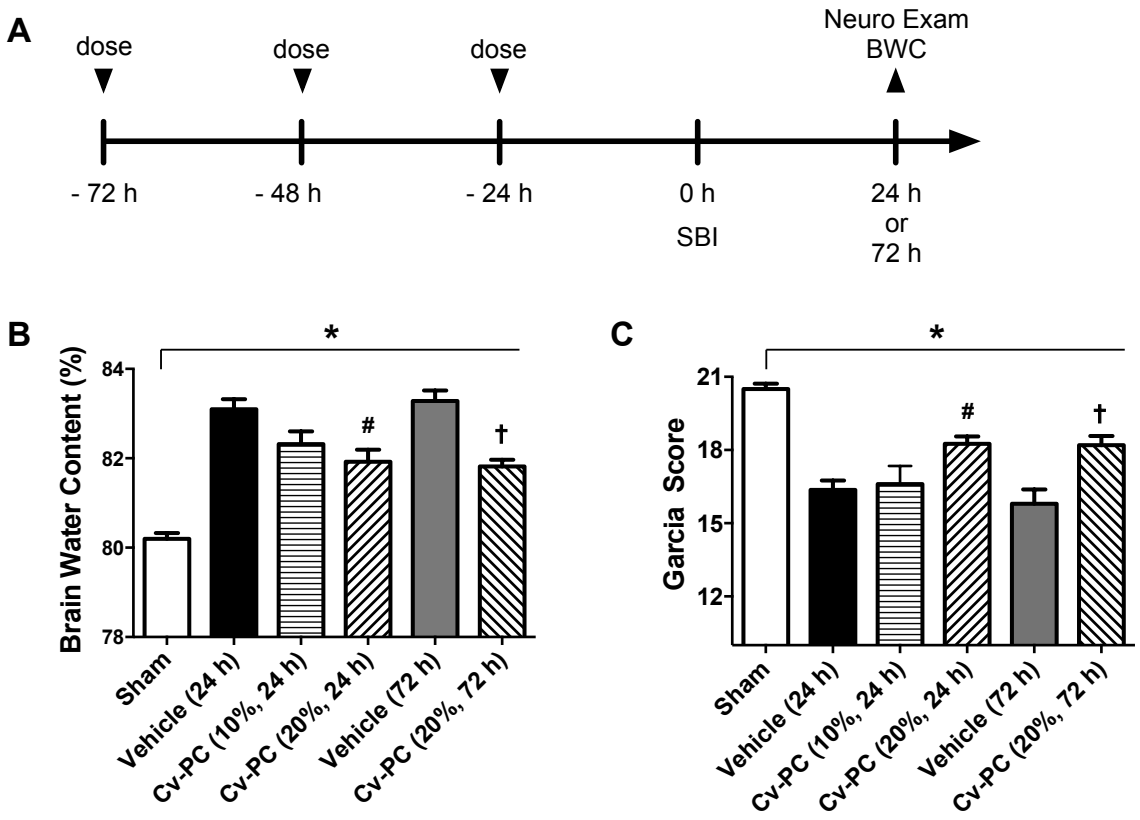

Figure 3.1. Cv-PC reduces brain edema and improves neurological function in SBI. (A) Schematic timeline of treatments and outcomes; rats received three subcutaneous injections of vehicle (normal saline) or Cv-PC doses (10% or 20% of the LD50 dose) at 72 h, 48 h, 24 h prior to surgery. (B) Cv-PC (20% LD50 dose) significantly reduced brain water content at 24 h and 72 h after SBI compared to those of vehicle-treated animals. (C) Cv-PC (20% LD50 dose) improved modified Garcia scores at 24 h and 72 h after SBI compared to those of vehicle-treated animals. * $p<0.05$ vs sham # $p<0.05$ vs vehicle (24 h), † $p<0.05$ vs vehicle (72 h). Data are shown as mean ± SEM, n=5-7 all groups. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

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 brain water content by Cv-PC (Figure 3.2B) to levels comparable to vehicle-treated animals. Additionally, COX-2 inhibition by NS398 reversed the protective effects of Cv-PC on neurological function at 24 h post-SBI (Figure 3.2C)

Cv-PC Reduces Expression of Inflammatory Mediators COX-2 and PGE_2 in 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 3.3). 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 3.4).

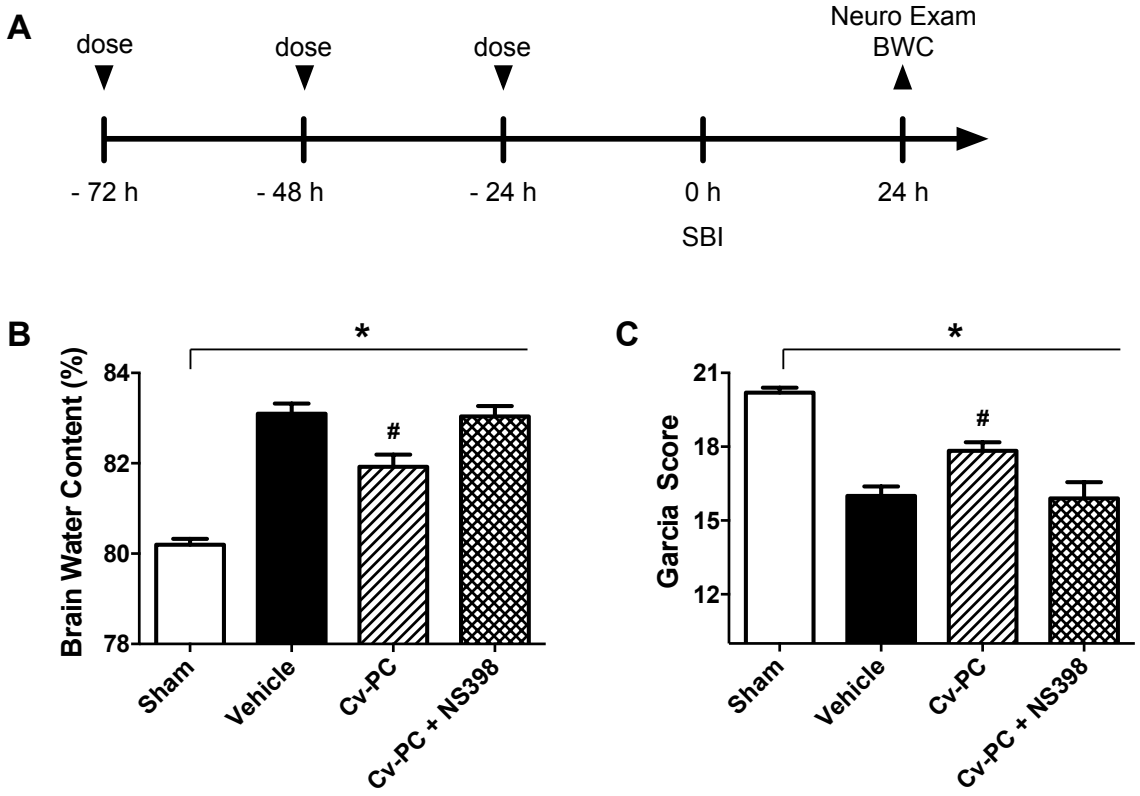

Figure 3.2. COX-2 Inhibition Reverses Cv-PC Effects. (A) Schematic timeline of treatments and outcomes; rats received three subcutaneous injections of vehicle (normal saline) or Cv-PC doses (20% LD50 dose) at 72 h, 48 h, 24 h prior to surgery. (B) Treatment with NS398 (COX-2 inhibitor, 10 mg/kg) prior to Cv-PC administration abolished the protective effects on brain water content. (C) Treatment with NS398 prior to Cv-PC administration reversed Cv-PC improvement of modified Garcia neurological scores. * $p<0.05$ vs sham, # $p<0.05$ vs vehicle. Data are shown as mean ± SEM, n=5-7 all groups. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

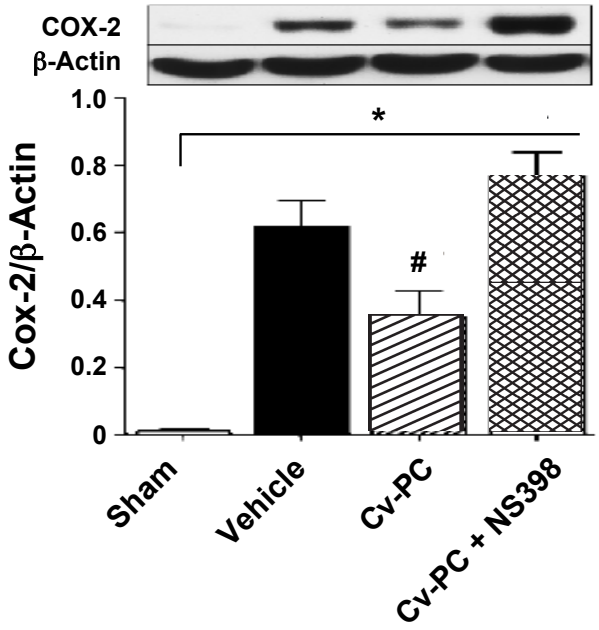

Figure 3.3. COX-2 Western Blot Analysis. COX-2 expression was significantly decreased after Cv-PC. Co-administration of Cv-PC and NS398, a COX-2 inhibitor reversed this effect. * $p<0.05$ vs Sham, # $p<0.05$ vs Vehicle. Data are shown as mean ± SEM, n=5/group. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

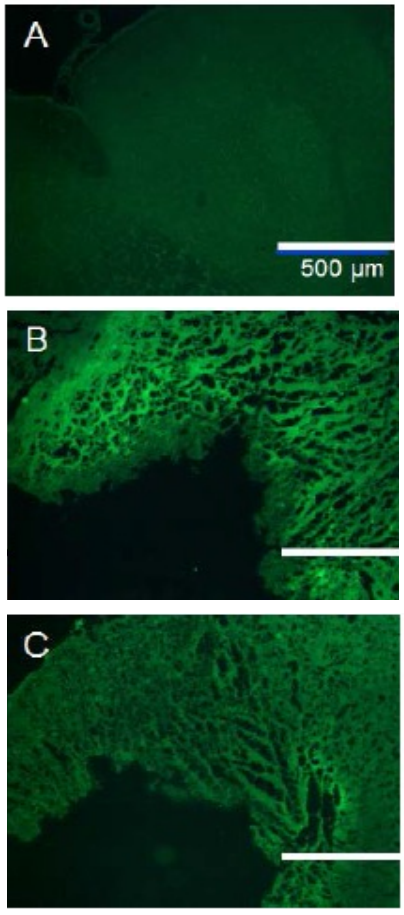

Figure 3.4. Immunohistological staining of ipsilateral frontal lobe tissue near the resection site using inflammatory marker PGE_2 (green). (A) Sham. (B) Vehicle. (C) Cv-PC. Cv-PC attenuated PGE_2 expression compared to that of the vehicle group. n=2/group.

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. We observed that Cv-PC reduces brain water content by approximately 1.2% and improves neurological function after SBI. Our results suggest that this neuroprotection is conferred through the PLA_2/COX-2 pathway, as inhibition of COX-2 reversed the protective effects of Cv-PC.

Our results correspond to other SBI rodent studies that indicate that the brain water content of tissue surrounding the resection site is increased by 3-4% during the first three days post-surgery (Matchett *et al.* 2006; Yamaguchi *et al.* 2007). At first glance, this 4% increase in brain water content may seem trivial, yet this extra 4% water content translates into a 25% increase in tissue volume, leading to high intracranial pressure, hypoperfusion of neurons, and cell death (Marmarou *et al.* 2000; Keep *et al.* 2012). Additionally, our results are congruent with previous studies with HBO-PC, which has been shown to attenuate COX-2 expression and brain water content, and improve functional outcome (Jadhav *et al.* 2009). Despite its promising results, HBO-PC continues to be limited by the lack of congruity in dosing regimens and the potential for oxygen toxicity. Like HBO-PC, Cv-PC appears to mitigate SBI brain edema and neurological injury by

tempering the expression of COX-2. Further purification and characterization of *C. helleri* proteins are needed to explore the clinical feasibility of Cv-PC.

Materials and Methods

Animal Experiments

All animals were housed in cages on a constant 12-hour light/dark cycle with controlled temperature and were given food and water ad libitum. 65 male Sprague Dawley rats (280-330g) were divided to the following treatment groups—sham, vehicle, Cv-PC, Cv-PC+NS3983 (COX-2 inhibitor, Abcam, ab120295). Vehicle and Cv-PC animals received either three daily subcutaneous doses of saline or *C. helleri* venom (20% of the LD50; 0.36 mg/kg) at 72 h, 48 h, and 24 h prior to blood draw or surgery. Sham animals received craniotomy only. SBI animals underwent craniotomy and a partial right frontal lobe resection 1mm above the horizontal line from bregma and 2mm to the right of the vertical line from bregma down to the skull base as previously described (Matchett, Hahn et al. 2006, Jadhav, Solaroglu et al. 2007). In Cv-PC+NS398 animals, NS398 (10 mg/kg, Abcam) was administered intraperitoneally one hour prior to Cv-PC doses, as previously described (Jadhav, Ostrowski et al. 2009)

Neurological Testing

A blinded observer tested all surviving animals 24 h after brain injury, using a modified 21-point sensorimotor test that measures 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 (Garcia, Wagner et al. 1995)

Brain Water Content

After neurological testing, animals were 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, Liu et al. 2004).

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).

Statistical Analysis

All values are mean ± SEM. GraphPad Prism software was used for statistical analysis. As specified in each figure legend, 1-way ANOVA, with

Tukey's comparisons, was used to determine differences among 3 or more. A *P* value <0.05 was considered significant.

Study Approval

All animal husbandry and procedures were approved by the Institutional Animal Care and Use Committee.

Acknowledgements

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CHAPTER FOUR

CROTALUS ATROX VENOM PRECONDITIONING INCREASES PLASMA FIBRINOGEN AND REDUCES PERIOPERTIVE HEMORRHAGE IN SURGICAL BRAIN INJURY RATS

Introduction

Perioperative bleeding is a serious complication in patients undergoing neurosurgical procedures (Bruder and Ravussin 1999, Seifman, Lewis et al. 2011). Intraoperative hemorrhage lengthens and complicates surgery and may trigger further injury by tissue hypo-perfusion or disruption of the blood-brain barrier (Bruder and Ravussin 1999, Sulejczak, Grieb et al. 2008). Postoperative hemorrhage poses a significant threat, given the finite nature of the intracranial space. Even small hematomas may result in increased intracranial pressure and brain herniation. Thus, the optimization of perioperative hemostasis in neurosurgical patients is of paramount importance.

The surgical brain injury (SBI) rodent model has been established to mimic injury during brain resection (Matchett, Hahn et al. 2006, Jadhav, Solaroglu et al. 2007). Given the elective nature of most neurosurgical procedures, the surgical brain injury (SBI) model is an ideal platform for preconditioning (PC) modalities, which are preemptive therapies in which mildly harmful stimuli are administered to induce endogenous protective mechanisms prior to major injury. PC has shown to be protective in the SBI model (Jadhav, Ostrowski et al. 2009, Jadhav, Ostrowski et al. 2010), as well as in many other injury models (Wada, Ito et al. 1996, Kapinya, Lowl et al. 2002, Nie, Xiong et al.

2006, Sang, Cao et al. 2006, Peng, Ren et al. 2008, Costa, Constantino et al. 2010).

Fibrinogen, an acute phase reactant with a half-life of 3.74 days (Martinez, Holburn et al. 1974), is part of the coagulation cascade and provides the substrate for fibrin clot formation. During coagulation, thrombin cleaves fibrinogen, producing soluble fibrin monomers that are able to be cross-linked by factor VIIIa to form a network on which a clot is built; fibrinogen also plays a role in platelet aggregation (Franchini and Lippi 2012). Decreased plasma fibrinogen levels have been identified as a potential modifiable risk factor for perioperative bleeding in cardiothoracic and orthopedic surgeries (Ucar, Oc et al. 2007, Carling, Jeppsson et al. 2011, Galas, de Almeida et al. 2014, Pillai, Fraser et al. 2014, Walden, Jeppsson et al. 2014). Lower perioperative fibrinogen levels, even within the normal reference range, have been shown to be associated with an increased risk of postoperative bleeding complications in patients undergoing elective intracranial surgery (Gerlach, Tolle et al. 2002, Adelmann, Klaus et al. 2014).

The hemostatic properties of snake venom toxins have long been studied for potential uses in clinical medicine. Some are used to detect coagulative disorders in the medical laboratory, while others have been examined for treating patients with coagulopathies (Marsh 2001, Earl, Masci et al. 2012). *Crotalus atrox* rattlesnake venom contains snake venom metalloproteinases (SVMPs) that cleave fibrinogen into fibrin split products (FSPs) in such a way that renders the proteins useless in fibrin polymerization (Pandya, Rubin et al. 1983, Abou-Saleh,

Connell et al. 2009). FSPs have been previously shown to increase fibrinogen plasma levels by inducing endogenous production of fibrinogen in hepatocytes (Fuller, Otto et al. 1985, Princen, Moshage et al. 1985, Amrani 1990). The unique degradation of fibrinogen by *C. atrox* SVMPs—creating FSPs without the induction of clotting—presents an opportunity to harness the toxic nature of the venom to elicit an endogenous response to increase plasma fibrinogen levels prior to elective surgery. We hypothesize that *C. atrox* venom preconditioning (Cv-PC) prior to surgery will increase plasma fibrinogen, thereby decreasing perioperative bleeding in the rodent surgical brain injury model.

Results

Cv-PC Reduces Intraoperative Blood Loss In Vivo

SBI resulted in an intraoperative hemorrhage volume of 1475 ± 75 µL which was significantly higher than that observed in sham animals (188 ± 23 µL). Twenty-four hours after Cv-PC, a dose-dependent reduction in intraoperative hemorrhage by 17.2% to 35.0% in Cv-PC-treated animals subjected to SBI—1221± 70 µL, 991 ± 62 µL, and 958 ± 75 µL in the 10%, 20%, and 30% LD50 doses, respectively (Figure 4.1B). Cv-PC with doses of 20% and 30% LD50 both significantly attenuated intraoperative hemorrhage compared to that of SBI animals given saline only (vehicle).

Cv-PC Reduces Postoperative Hematoma In Vivo

Next, the effect of Cv-PC on the development of postoperative hematoma

in the brain parenchyma was examined. Assessed at 24 h after SBI surgery, the postoperative hematoma demonstrated a dose-dependent decrease in volume by 48.7% to 64.9% for Cv-PC which was significantly decreased for all doses compared to that of SBI animals receiving vehicle preconditioning (Figures 4.1C). The postoperative hematoma volume in vehicle-treated animals was 22.5 ± 1.6 μL, and that of Cv-PC-treated animals was 11.3 ± 1.2 μL, 8.0 ± 0.7 μL, and 7.9 ± 0.5 μL in the 10%, 20%, and 30% LD50 doses, respectively.

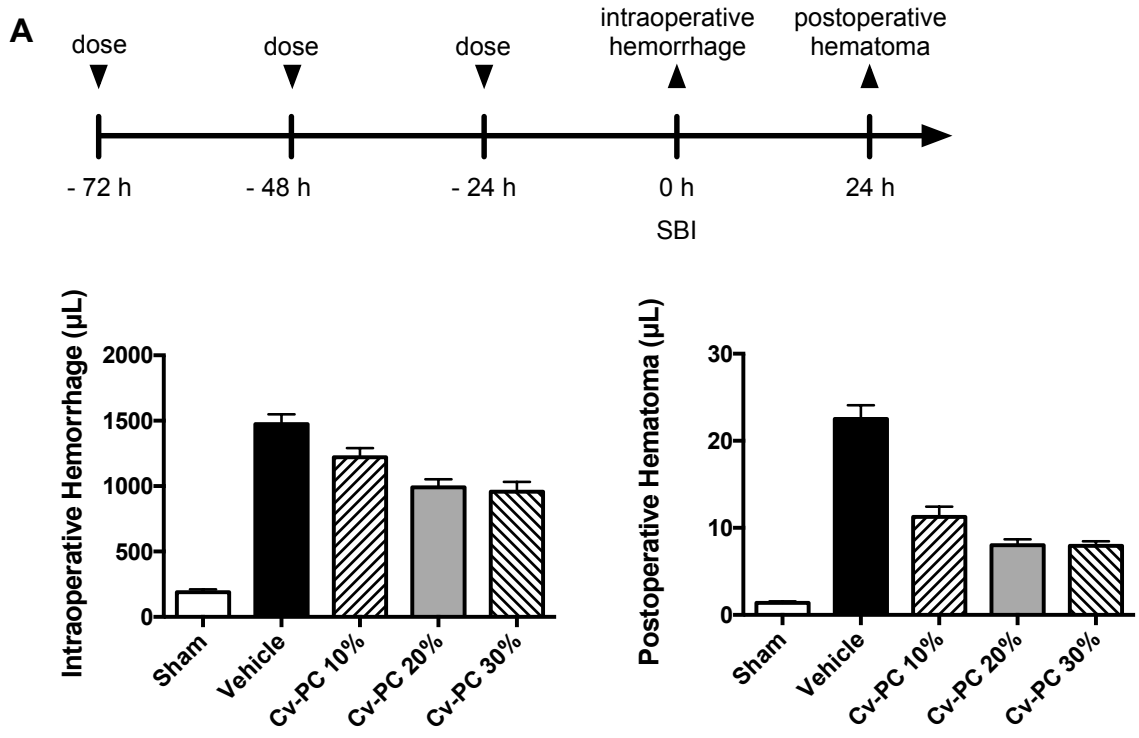

Figure 4.1. Cv-PC reduces intraoperative hemorrhage and postoperative hematoma in a dose-dependent manner in SBI. (A) Schematic timeline of treatments and outcomes; treatment group rats received three subcutaneous doses of vehicle (normal saline) or Cv-PC (10%, 20%, and 30% of the LD50 dose) at 72 h, 48 h, 24 h prior to surgery; sham animals received craniotomy only; intraoperative hemorrhage volume was collected throughout surgery and postoperative hematoma was collected at 24 h post-surgery. (B) Intraoperative hemorrhage and (C) postoperative hematoma volumes were assessed by spectrophotometric hemoglobin assay; both were reduced by subcutaneous Cv-PC in a dose-dependent manner. Cv-PC: Crotalus atrox venom preconditioning; SBI: surgical brain injury. * $p<0.05$ vs Sham, # $p<0.05$ vs Vehicle. Data are shown as mean ± SEM, n=5-6 all groups. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

Cv-PC Increases Plasma Fibrinogen and Plasma FSPs In Vivo

To assess the hematologic effects of Cv-PC, coagulation parameters were measured following administration of vehicle or Cv-PC at 24 h following the third dose of Cv (corresponding to the intended surgery time) or at 6 h post-surgery in animals that received SBI (Figure 4.2A). Animals that received Cv-PC showed significantly increased plasma fibrinogen at time of surgery, as well as at 6 h postoperatively (Figure 4.2B, Table 4.1). By time of surgery, Cv-PC raised plasma fibrinogen by an average of 78.4 mg/dL—a 31.6% increase in concentration. At 6 h post-SBI, the fibrinogen remained higher in the Cv-PC-treated animals than that of Vehicle treated animals. FSPs were only detected in the plasma of Cv-PC-treated animals.

PT, INR, PTT, D-Dimer, and Soluble Fibrin Remain Normal After Cv-PC

To assess Cv-PC's potential for inducing coagulopathic events we measured PT, INR, PTT, D-Dimer, and soluble fibrin. We did not observe clinically significant changes in these parameters following Cv-PC (Table 4.1).

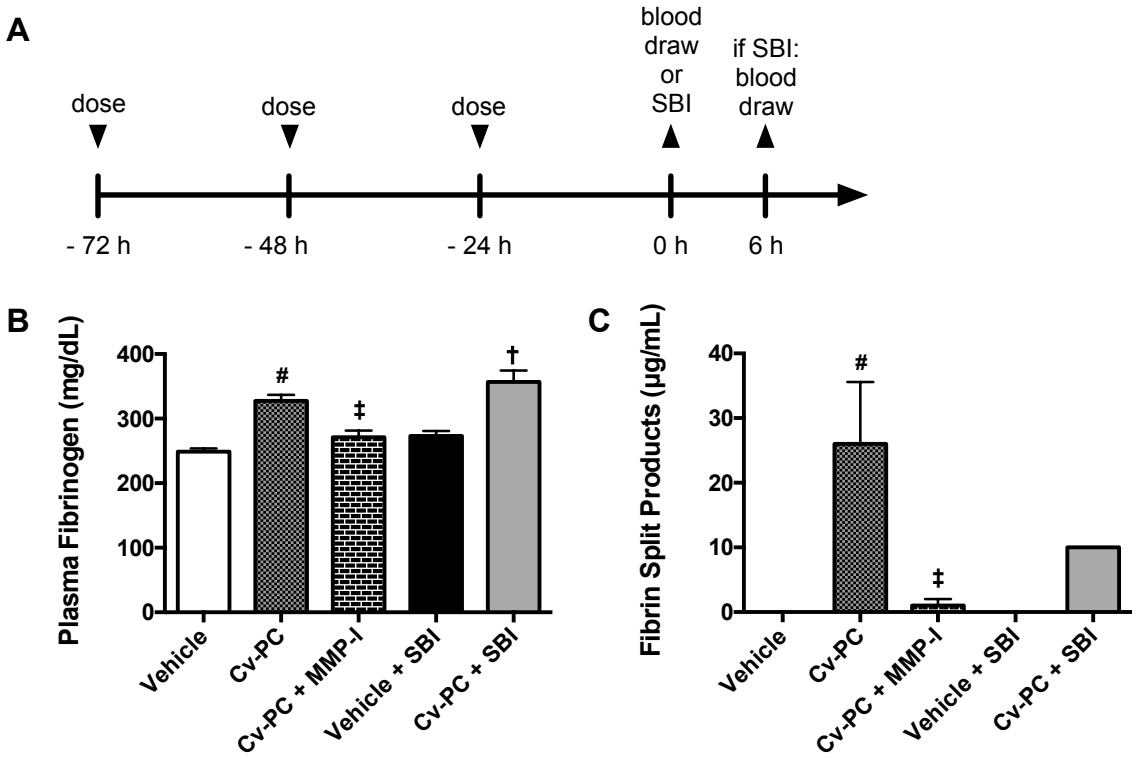

Figure 4.2. Cv-PC increases plasma fibrinogen and generates fibrin split products. (A) Schematic timeline of treatments and outcomes; rats received three subcutaneous injections of vehicle (normal saline), Cv-PC doses (20% of the LD50 dose), or Cv-PC doses incubated with matrix metalloproteinase inhibitor (MMP-I; with equal concentrations of Marimastat and AG-3340 to Cv) at 72 h, 48 h, 24 h prior to blood draw by cardiac puncture or surgery; animals undergoing surgery received blood draws at 6 h post-surgery. (B) Cv-PC significantly increased plasma fibrinogen at the time of surgery and at 6 h post-surgery. (C) Fibrin split products are only detectable in the plasma of Cv-PC-treated animals. MMP inhibition suppressed formation of FSPs by Cv-PC. # $p<0.05$ vs Vehicle, † $p<0.05$ vs Vehicle+SBI, ‡ $p<0.05$ vs Cv-PC. Data are shown as mean ± SEM, n=8-12 all groups. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

Table 4.1. *Coagulation parameters after Cv-PC.*

	Vehicle	Cv-PC	Cv-PC +MMP-I	Vehicle+SBI	Cv-PC+SBI
n =	12	10	10	10	8
Fibrinogen (mg/mL)	248.9 ± 4.9	327.6 ± 9.1*	271.0 ± 10.6	273.2 ± 7.6	356.9 ± 17.6*
FSPs (µg/mL)	0.0 ± 0.0	26.0 ± 9.6	1.0 ± 1.0	0.0 ± 0.0	10.0 ± 0.0
PT (sec)	10.67 ± 0.05	10.52 ± 0.05	10.34 ± 0.08	10.25 ± 0.10	10.04 ± 0.07
INR	0.97 ± 0.01	0.92 ± 0.01	0.93 ± 0.01	0.91 ± 0.01	0.90 ± 0.00
PTT (sec)	15.07 ± 0.15	14.76 ± 0.04	15.32 ± 0.38	15.16 ± 0.12	14.83 ± 0.04
D-Dimer (µg/mL)	0.03 ± 0.01	0.0 ± 0.0	0.04 ± 0.02	0.01 ± 0.01	0.06 ± 0.01
Soluble Fibrin (ng/mL)	41.8 ± 8.9	65.6 ± 8.1	48.2 ± 10.2	39.5 ± 6.7	80.8 ± 11.3*

Data are shown as mean ± SEM. Cv-PC, *C. atrox* venom preconditioning; FSPs, fibrin split products; INR, international normalized ratio; MMP-I, metalloproteinase inhibitor; PTT, partial thromboplastin time; PT, prothrombin time; SBI, surgical brain injury. * $p<0.05$ vs Vehicle.

PC with Several Cv Fractions Reduces Intraoperative Hemorrhage and Postoperative Hematoma Volumes In Vivo

Whole Cv and its fractions from gel filtration separation were separated by reversed-phase HPLC (Figures 4.3A and 4.3B), and fraction components of all substantial peaks were identified by LC-MS. Gel filtration Cv fractions 1, 2, 3, 4, 5 and pooled fractions 6-8 were then used for Cv-PC. Cv-PC with fractions 2, 3, 4, and 5 improved hemorrhage volumes.

MMP Inhibition Blocks Cv-PC Effects on Hemorrhage Volumes, Plasma Fibrinogen and FSPs

To evaluate the role of SVMPs in Cv-PC against SBI, two MMP inhibitors were coadministered with Cv for each injection during Cv-PC. The concomitant administration of MMP inhibitors abolished the increase in plasma fibrinogen by Cv-PC (Figure 4.2B, Table 4.1) to levels similar to that of vehicle PC animals. Additionally, Cv-PC administered with MMP inhibitors significantly diminished the generation of FSPs (Figure 4.2C, Table 4.1). Furthermore, SVMP inhibition by MMP inhibitors had similar intraoperative hemorrhage and postoperative hematoma volumes to those of vehicle treated animals, which were significantly higher than those of Cv-PC animals (Figure 4.4B, 4.4C).

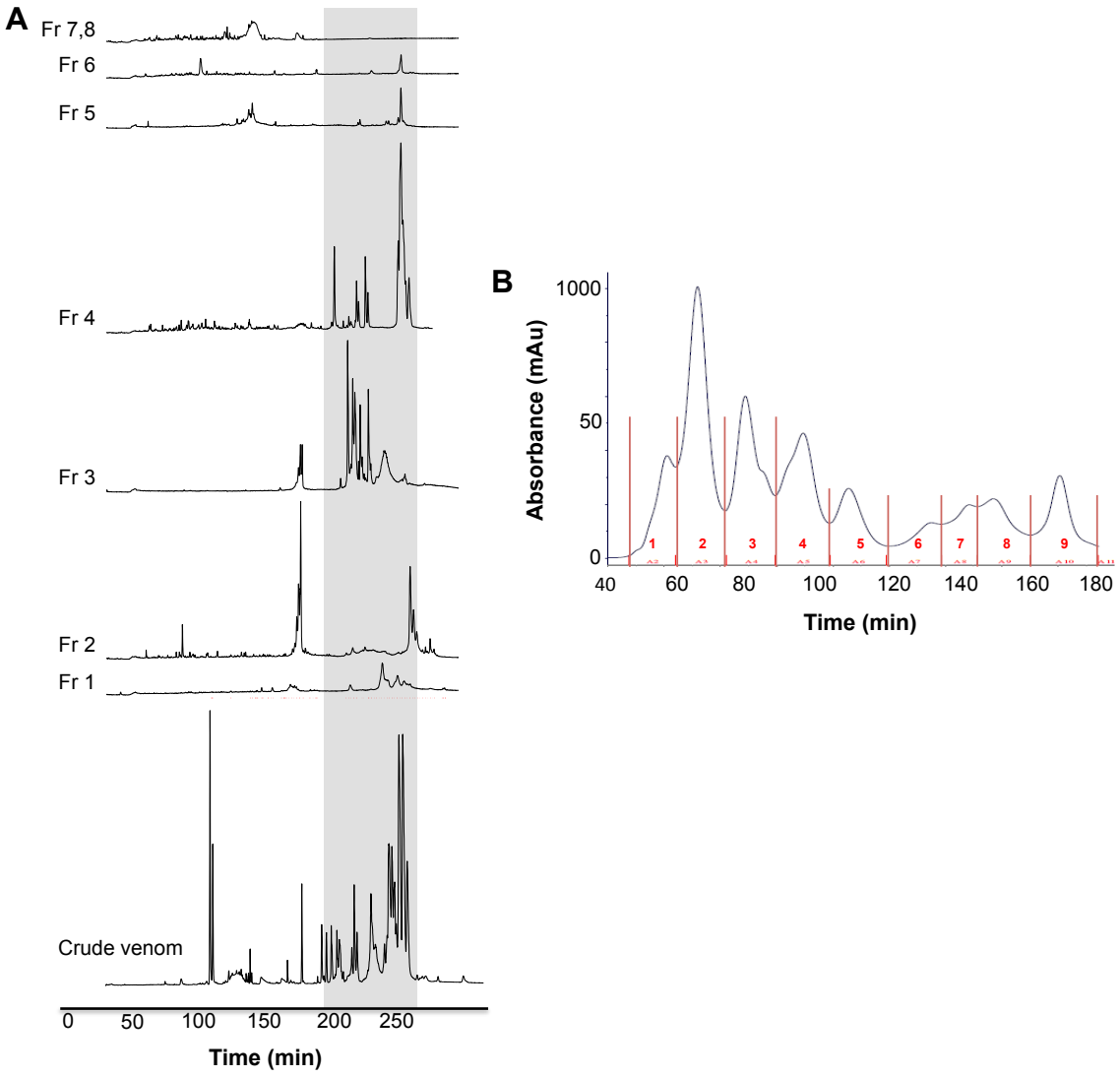

Figure 4.3. Reversed-Phase HPLC and Gel Filtration Chromatographs of *C. atrox* Venom. (A) Reversed-Phase HPLC of crude *C. atrox* venom (Cv) and Cv fractions (Fr) collected from gel filtration chromatography; absorbance at 214 nm. Grey demarcates the range in which snake venom metalloproteinases (SVMPs) represent the prominent Cv component as identified by LC-MS (B) Gel Filtration Chromatograph of Cv fractionation. Collected fractions are labeled in red numbers.

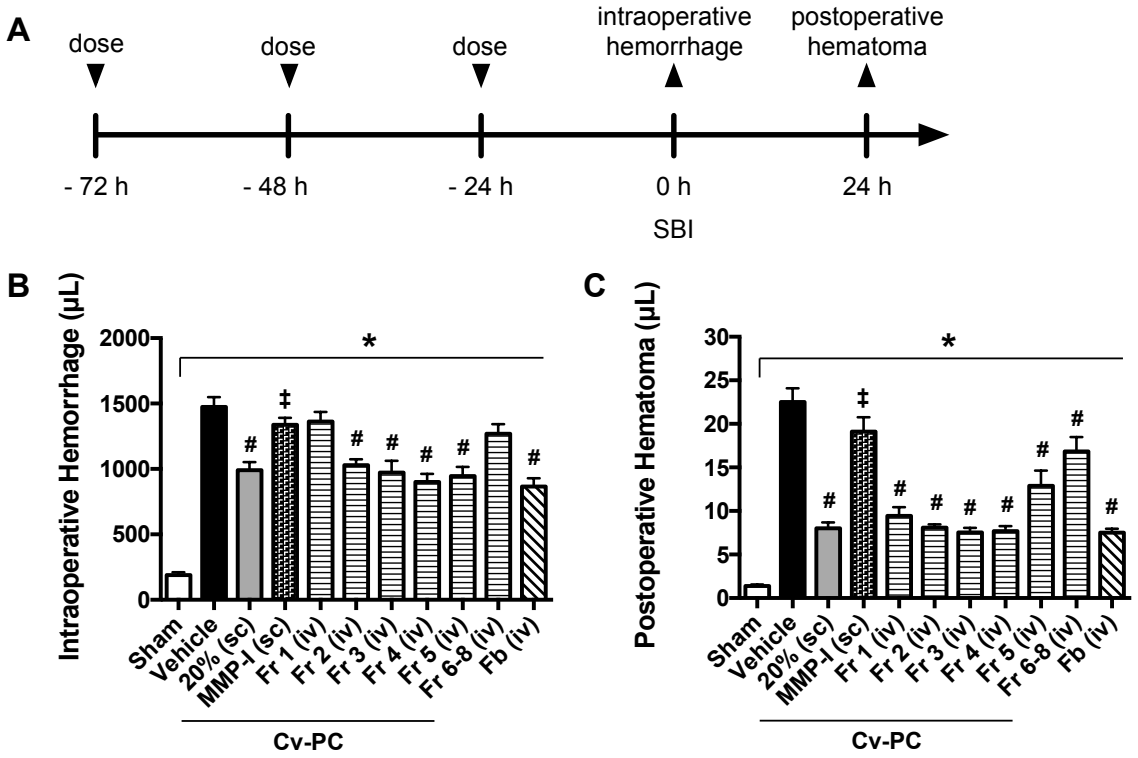

Figure 4.4. Cv-PC intraoperative hemorrhage and postoperative hematoma in SBI. (A) Schematic timeline of treatments and outcomes; rats received three subcutaneous doses of vehicle (normal saline), Cv-PC (20% of the LD50 dose), Cv-PC doses incubated with matrix metalloproteinase inhibitor (MMP-I; with equal concentrations of Marimastat and AG-3340 to Cv), or fractions (Fr) of Cv at 72 h, 48 h, 24 h prior to surgery. Sham animals received craniotomy only. Fibrinogen-treated animals received one dose of fibrinogen (Fb,15mg/1.5mL) 15 min before surgery. Routes of dose administration were either subcutaneous (sc) or intravenous (iv). Intraoperative hemorrhage volume was collected throughout surgery and postoperative hematoma was collected at 24 h post-surgery. (B) Intraoperative hemorrhage volume was significantly reduced by Cv-PC (20% LD50) and Cv-PC by Fr 2, 3, and 4. MMP inhibition during Cv-PC reversed the effect. Direct administration of fibrinogen reduced intraoperative hemorrhage. (C) Postoperative hematoma volume was significantly decreased by Cv-PC (20% LD50) and Cv-PC by all fractions. MMP inhibition during Cv-PC reversed the effect. Direct administration of fibrinogen reduced the development of postoperative hematoma. * $p<0.05$ vs Sham, # $p<0.05$ vs Vehicle, ‡ $p<0.05$ vs Cv-PC (20% LD50). Data are shown as mean ± SEM, n=5-6 all groups. 1-way ANOVA, with Tukey's comparisons, was used to determine differences.

Direct Fibrinogen Administration Reduces Intraoperative Hemorrhage and Postoperative Hematoma Volumes In Vivo

To determine the effect of direct fibrinogen administration on SBI pathophysiology, 15 mg/1.5 mLof fibrinogen was administered 15 min prior to induction of surgery. In fibrinogen-treated animals, a reduction of intraoperative hemorrhage (Figure 4.4B) and postoperative hematoma volumes (Figure 4.4C) were observed comparable to those of Cv-PC-treated animals.

C. atrox Venom Causes Dose-dependent Hemostatic Changes in Human Whole Blood In Vitro

To begin assessing the clinical feasibility of Cv-PC, the direct effects of Cv on whole human blood was evaluated for clotting, sticking, and clumping parameters. No change in any of these measures was found for saline; however, a dose-dependent increase in all three parameters was observed at 2 μg/mL, 4 μg/mL, and 8 μg/mL doses of Cv.

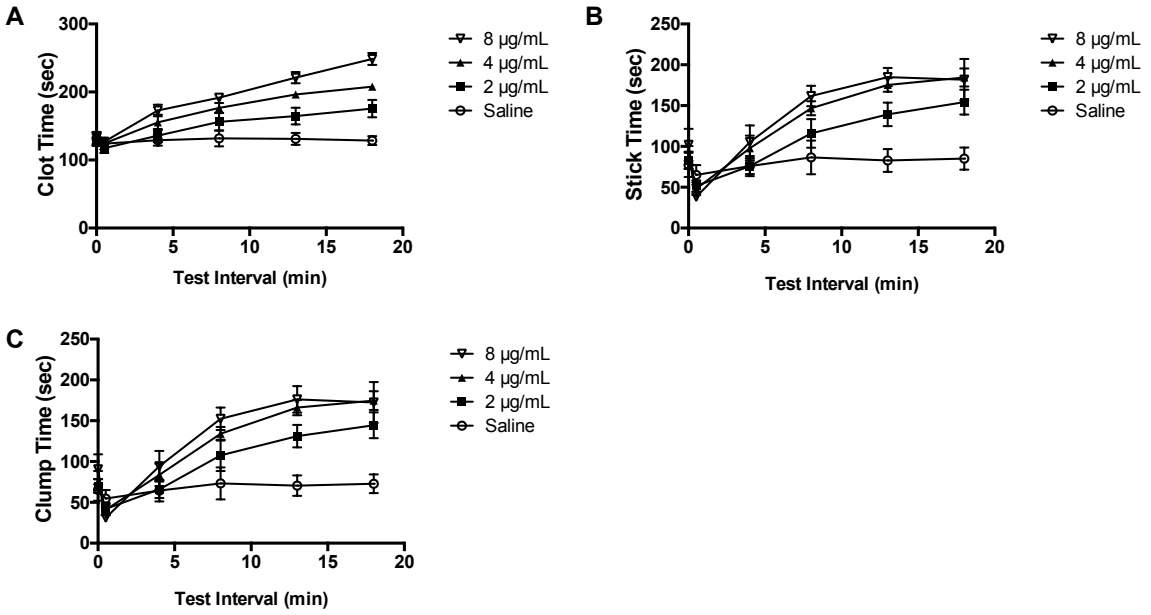

Figure 4.5. Dose-dependent effects of *C. atrox* venom on human whole blood *in vitro*. Minimal stimulation clotting/platelet testing imitates *in vivo* clotting on a sample of whole blood and measures three parameters—clot, clump, stick times. Clot time correlates with fibrinogen, intrinsic / extrinsic pathway function, whereas clump and stick times correlate well with platelet function. We observed a dose-dependent increase in (A) Clotting time (B) Stick time (C) Clump time at (2, 4, and 8 µg/mL of *C. atrox* venom. Data are shown as mean ± SEM, n=3 all groups.

Discussion

Perioperative hemorrhage is a devastating complication in neurosurgery (Bruder and Ravussin 1999, Seifman, Lewis et al. 2011), making optimization of hemostasis of the utmost importance. This study uses a novel preemptive approach for improving perioperative hemostasis by eliciting endogenous mechanisms using *C. atrox* venom preconditioning. Our hypothesis was that Cv-PC would attenuate the hemorrhage induced by SBI via an increase of endogenous fibrinogen production. First, we observed that Cv-PC reduces intraoperative blood loss and postoperative hematoma by more than 30% in the SBI rodent model. Second, while coagulation was improved by Cv-PC, coagulation parameters—PT, INR, PTT, D-Dimer, and soluble fibrin—showed no indication that Cv-PC caused a thrombotic state. Third, our results point to SVMP as the active component of Cv that cleaves fibrinogen into FSPs and imparts the protective effects of Cv-PC; and inhibition of SVMP reversed the effects of Cv-PC. Finally, preconditioning with Cv fractions which primarily contain SVMP retained the effects of Cv-PC by crude venom.

Choice of Crotalus *Venom*

Given the ability of *Crotalus* venoms to generate FSPs without the initiation of clotting (Pandya, Rubin et al. 1983, Abou-Saleh, Connell et al. 2009) and the previously demonstrated induction by FSPs of hepatocyte fibrinogen production (Fuller, Otto et al. 1985, Princen, Moshage et al. 1985, Amrani 1990), *C. adamanteus, C. atrox,* and *C. viridis helleri* venoms were considered for PC.

We elected to use *C. atrox* venom because it has been demonstrated to have strong fibrinolytic activity with no detectable fibrinogen clotting activity, whereas the other *Crotalus* venoms were measured to have a some fibrinogen clotting activity (Bajwa, Markland et al. 1981). To determine the optimal dose of venom that can elicit therapeutic benefit to animals subjected to SBI, a dose dependent study was conducted in which we administered three daily doses of Cv (10%, 20%, and 30% of the LD50; 1.85 mg/kg, 3.7 mg/kg, and 5.55 mg/kg, respectively) with the last dose 24 h prior to surgery. The results of the dose dependent study suggest that the effectiveness of Cv-PC for reducing intraoperative hemorrhage and postoperative hematoma volumes plateaus at the 20% LD50 dose. Additionally, these data suggest that Cv-PC is viable treatment to improve hemostasis in the SBI neurosurgical model.

Effects of Cv on Coagulation

Prothrombrin time (PT), international normalized ratio (INR), and partial thromboplastin time (PTT) are routinely assessed to evaluate the extrinsic and intrinsic pathways of the coagulation cascade. Increased INR or prolongation of PT or PTT is suggestive of bleeding diathesis. Shortened PTT may be associated with increased risk of venous thromboembolism (Tripodi, Chantarangkul et al. 2004). D-Dimer and soluble fibrin levels are used to screen for disseminated intravascular coagulation (DIC), which is characterized by systemic activation of blood coagulation and subsequent formation of microvascular thrombi that may ultimately lead to severe bleeding by

consumptive depletion of coagulation factors and/or platelets (Levi and Ten Cate 1999). The findings that Cv-PC had no clinically significant effect on any of these parameters supports previous findings that *C. atrox* venom has limited clotting activity (Bajwa, Markland et al. 1981).

Identifying the Role of SVMP in PC Against SBI

Initially, Cv was fractionated by reverse-phase chromatography, which may denature proteins, and is therefore unsuitable for assays of biological activity; thus, gel filtration chromatography, which preserves protein activity but achieves much lower resolution in protein separation than reversed-phase chromatography was utilized to obtain 9 fractions. These fractions were administered intravenously to assure intravascular exposure to the Cv components. In both intraoperative and postoperative hemorrhage volumes, fractions 2, 3, and 4, which contain primarily SVMPs (Figure 3), provided improved hemostasis most consistently.

To further elucidate SVMP as the active protein responsible for increased homeostasis during SBI, we evaluated the effects of blocking SVMP during Cv-PC. Since specific inhibitors of SVMP are not available, we elected to use commercially available MMP inhibitors, Marimastat and AG-3340, which have been previously described to block other SVMPs (Howes, Theakston et al. 2007). Our findings, that coadimistration of MMP inhibitors and Cv during PC prevents Cv-PC benefits, suggest that SVMP plays a crucial role in the generation of FSPs and fibrinogen, as well as in the improved hemostasis conferred by Cv-PC.

Investigation of the Role of Fibrinogen in Cv-PC

To determine of fibrinogen was the key mediator of Cv-PC effect after SBI, fibrinogen treatment was given 15 minutes before SBI (15 mg/1.5 mLof fibrinogen). This dose was determined by computing the required amount of protein to raise plasma fibrinogen by approximately 75 mg/dL, given the estimated blood volume of the animals, as previously published (Diehl, Hull et al. 2001). Our results showed that this dose of fibrinogen, which correlates with the increase in fibrinogen concentration provided by Cv-PC, was capable of improving perioperative hemostasis.

Clotting, Clumping, and Sticking Tests

Minimal stimulation clotting/platelet testing, which imitates *in vivo* clotting on a sample of whole blood, measures three parameters—clot, clump, and stick times. Clot time correlates with fibrinogen, intrinsic / extrinsic pathway function, whereas clump and stick times correlate well with platelet function. A dose dependent reduction in all three parameters demonstrates impaired clotting function by Cv. The prolonged clot time likely represents the depletion of fibrinogen by SVMP cleavage. The prolonged clump and stick times indicate decreased platelet function, which likely results from fibrinogen depletion, as platelets are supported by fibrinogen to mediate adhesion and aggregation (Coller, Peerschke et al. 1983, Ikeda, Handa et al. 1991, Savage and Ruggeri 1991).

Clinical Application of Increasing Plasma Fibrinogen Levels Before Major Elective Surgery

Our findings are congruent with the growing body of literature that emphasizes plasma fibrinogen level as a potential modifiable risk factor for perioperative hemorrhage (Gerlach, Tolle et al. 2002, Ucar, Oc et al. 2007, Carling, Jeppsson et al. 2011, Adelmann, Klaus et al. 2014, Galas, de Almeida et al. 2014, Pillai, Fraser et al. 2014, Walden, Jeppsson et al. 2014). These studies indicate that lower fibrinogen, even within normal limits, are correlated with increased bleeding and that higher levels of fibrinogen are associated with better outcomes. Fibrinogen is fundamental to effective clot formation. It circulates at the highest concentration of all the coagulation factors (Lowe, Rumley et al. 2004) and is the first coagulation factor to drop to critically low levels during major hemorrhage. Dilutional deficiency of fibrinogen develops earlier than any other hemostatic abnormality when plasma-poor red blood cell preparations are used to replace blood loss (Hiippala, Myllyla et al. 1995). Yet in current practice, the options for increasing plasma fibrinogen are limited to transfusions of fibrinogen-containing preparations like fresh frozen plasma, cryoprecipitate, and fibrinogen concentrate. Fresh frozen plasma (FFP) varies in fibrinogen concentration as it is collected from donors. The concentration of fibrinogen in FFP reportedly ranges between 100-300 mg/dL (Theusinger, Baulig et al. 2011). Because of these concentrations, large volumes of FFP are necessary for even modest increases in plasma fibrinogen. Cryoprecipitate, obtained by concentrating FFP, has about 200 mg of fibrinogen per unit at a concentration of

about 550 mg/dL (Lee, Lee et al. 2014). Fibrinogen concentrate, manufactured from human plasma and available as a pasteurized, lyophilized powder, is given at a concentration of 2000 mg/dL (Levy, Szlam et al. 2012). A previous study determined that in order to raise plasma fibrinogen levels from 150 mg/dL to 170 mg/dL, 14 units of FFP, 8 units of cryoprecipitate, or 2 units of fibrinogen concentrate is required (Collins, Solomon et al. 2014); thus, fibrinogen supplementation by FPP and cryoprecipitate administration may cause fluid overload. In addition, these transfusions are not without risk; they are associated with complications that include allergic reaction, infections, and transfusion-related acute lung injury (Busch, Glynn et al. 2005, Inaba, Branco et al. 2010).

An estimated 31% of elective neurosurgeries require blood transfusions (Bhatnagar 2007). A preventive therapy that reduces perioperative hemorrhage would decrease the need for blood transfusions, ultimately cutting perioperative costs; up to $6.03 million is spent on blood and transfusion-related care for surgical patients annually per hospital (Shander, Hofmann et al. 2010). While our study focused on Cv-PC in the SBI model, the implications of our results extend beyond the field of neurosurgery. Cv-PC could also be applied to elective surgeries in other fields, as perioperative hemorrhage is the bane of surgeons in every specialty. Over 200 million major elective surgeries are performed worldwide per year (Weiser, Regenbogen et al. 2008). Additional studies are needed to assess the translational feasibility of Cv-PC. Further isolation and characterization of proteins responsible are required to establish mechanism and safety. While our study demonstrated that Cv-PC did not result in coagulation

parameters indicative of a thrombotic state, more studies are needed to assess whether the Cv-PC increases the risk of pathologic thrombotic events.

Materials and Methods

Animal Experiments

All animals were housed in cages on a constant 12-hour light/dark cycle with controlled temperature and were given food and water ad libitum. 121 male Sprague Dawley rats (280-330g) were divided to the following treatment groups—sham, vehicle, Cv-PC (various doses and fractions), Cv-PC+MMP-I, fibrinogen. Vehicle and Cv-PC animals received either three daily subcutaneous doses of saline or *C. atrox* venom (10%, 20%, and 30% of the LD50; 1.85 mg/kg, 3.7 mg/kg, and 5.55 mg/kg, respectively, for the dose dependent study and 20% LD50 for the remainder of the experiments) at 72h, 48h, and 24h prior to blood draw or surgery. Animals were further divided into non-SBI or SBI groups; SBI animals underwent craniotomy and a partial right frontal lobe resection 1mm above the horizontal line from bregma and 2mm to the right of the vertical line from bregma down to the skull base as previously described (Matchett, Hahn et al. 2006, Jadhav, Solaroglu et al. 2007). In Cv-PC+MMP-I animals, Cv (3.7 mg/kg) was incubated with Marimastat (3.7 mg/kg, Sigma) and AG-3340 (3.7 mg/kg, Sigma) for 30 min at 37°C prior to subcutaneous injection, as previously described (Gutierrez, Leon et al. 1998, Howes, Theakston et al. 2007). Fibrinogen-treatment animals received one dose of fibrinogen (15 mg/1.5 ml, Sigma) 15 min before surgery. Blood draws on animals in the SBI groups were

performed 6 h after surgery. Coagulation studies performed by a blinded operator using standard human testing included aPTT, PT, INR, D-dimers, soluble fibrin, fibrinogen and fibrin split products (FSP). Previous studies have established normal ranges in rats using human test methods (Takahashi, Hirai et al. 2011).

Spectrophotometric Assay of Hemoglobin

At 24 h after SBI, the animals were placed under deep anesthesia and transcardially perfused with 0.1 M PBS until the outflowing fluid from the right atrium became colorless. The brain was removed and dissected into left and right hemispheres. Hematoma volume was quantified by spectrophotometric assay of brain hemoglobin content as described previously (Hu, Ma et al. 2011). A standard curve was obtained by adding incremental volumes of homologous blood to perfused brain tissue of naïve animals. The hemispheric samples were then homogenized and sonicated in distilled water followed by a 30-minute centrifugation (13,000 *g* at 4°C); Drabkin reagent (1.6 mL; Sigma) was added to 0.4 mL supernatant aliquots and optical density was measured at 540 nm via spectrophotometer (Spectronix 3000; Milton-Roy). Hemoglobin measurements were compared with the standard curve to obtain hemorrhage volume expressed as μL of blood per ipsilateral hemisphere. Intraoperative hemorrhage volume was collected by suction throughout surgery and was added to packing material used for hemostasis during the procedure; distilled water was added bringing the volume to 50 mL for each collected hemorrhage sample. A standard curve was obtained by adding incremental volumes of homologous blood to distilled water

to create solutions of 50 mL total volume. Samples were homogenized, sonicated, and prepared for spectrophotometric assay as described above. Testing was done in duplicate.

Blood Draws

Blood was collected by cardiac puncture. The animals were anesthetized with isoflurane, then moved to a sacrifice table where incision was made extending from right upper quadrant to left upper quadrant. Once inside the peritoneum the diaphragm was cut out to visualize the heart and a 18-gauge needle was placed in the right ventricle; the first few drops of blood we discarded to eliminate tissue factor from the blood draw. Further blood was collected in 10-cc syringe and rapidly transferred to the appropriate collection tubes, gently mixed by ten inversions, and delivered to the appropriate laboratories for subsequent testing.

Specimens destined for soluble fibrin, PT/INR, aPTT, fibrinogen and D-Dimer testing were anticoagulated in a mixing ratio of one part citrate to nine parts blood using Greiner Bio-One Vacuette® tubes (Part No. 454334) containing 3.2% (0.109M) Sodium Citrate Solution. Specimens for Fibrin Split Product (FSP) testing were added to Thrombo-Wellcotest™ Collection Tubes (Part No. R30853001) containing soya bean trypsin inhibitor (approximately 3600 NF units/tube) and *C. atrox* venom (>10µg/tube) for the collection of 2mL whole blood.

Soluble Fibrin Analysis

All samples were maintained at 37°C from draw through testing. The testing was as described by Hay et. al., but modified slightly since the appearance of endpoints in rat blood were somewhat atypical as compared to human endpoints (Hay and Bull 2002). The modification (reading endpoints using 90 µL plasma rather than the usual 150 µL whole blood) clarified the endpoints and allowed for comparison between rats and treatment groups. However, the results cannot be compared to usual human values.

PT/INR/APTT/Fibrinogen Analysis

Blood was centrifuged at 7200 RPM (4440 x g) for 2 min using the STATSpin Express 3 to obtain plasma for testing. Testing was performed using a BCS® XP System (Siemens Healthcare, Munich, Germany). The PT/INR were performed using Dade® Innovin® reagent. The aPTT was performed using Dade® Actin® FSL reagent and 0.025M $CaCl_2$. Fibrinogen assays were performed using Dade® Thrombin Reagent, Dade® Fibrinogen Standard and Dade® Owren's Veronal Buffer.

D-Dimer Analysis

Blood was centrifuged at 7200 RPM (4440 x g) for 2 min using the STATSpin Express 3 to obtain plasma for testing. Testing was performed using the Cobas® 8000 modular analyzer (Roche Diagnostics, Indianapolis, IN). The method is based on latex particles coated with monoclonal anti-human D-Dimer

antibodies (mouse) to which addition of a sample containing D-Dimer increases turbidity leading to changes in absorbance over time.

Fibrin Split Products Analysis

Blood was centrifuged at 7200 RPM (4440xg) for 2 min using the STATSpin Express 3 to obtain serum for testing. Testing was performed using the Thrombo-Wellcotest™ Rapid Latex FDP Assay. The kit test utilizes latex particles, which have been coated with rabbit antibodies to fibrin fragments. The approximate concentration is determined from agglutination observed on serial specimen dilutions.

HPLC

Lyophilized crude venom was diluted to a concentration of 6 mg/mL in Buffer A (0.065% TFA, 2% acetonitrile in Nanopure water) and centrifuged at 15,000 *g* for 10 min. The supernatant (100 µL) was fractionated on an ÄKTAmicro high-pressure liquid chromatography (HPLC) system (GE Healthcare Life Sciences, Piscataway, NJ, USA) fitted with two reversed-phase (RP) columns (SOURCE 5RPC ST polystyrene/divinyl benzene, 4.6 150 mm; GE Healthcare) run in series at a flow rate of 0.5 mL/min, using a linear gradient of 0–100% Buffer B (0.05% TFA, 80% acetonitrile in Nanopure water) over 40 column volumes. Protein elution was monitored at 214 nm using Unicorn 5.0 (GE Healthcare Lifesciences) software, and fractions were collected manually.

LC-MS

Each fraction was subjected to reduction and alkylation prior to enzymatic digestion using dithiothreitol and iodoacetamide, respectively, following the protocol outlined by Matsudaira (1993). Proteins were then digested with proteomics-grade porcine pancreatic trypsin (Sigma-Aldrich, St. Louis, MO, USA). We desalted samples using C18 ZipTips (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. We analyzed the desalted tryptic peptides with a ThermoFinnigan LCQ Deca XP spectrometer (ThermoFinnigan, Waltham, MA, USA) equipped with a PicoView 500 nanospray apparatus using Xcalibur software (ver. 1.3; ThermoFinnigan, Waltham, MA, USA) for instrument control and data acquisition. Separation was performed on a 10-cm x 75-m-i.d. C18 Biobasic bead column (New Objective, Woburn, MA, USA) by injecting 20-uL samples. Mobile phase B consisted of 98% acetonitrile, 2% water, and 0.1% formic acid. The gradient program was: 0% B at 0.18 mL/min for 7.5 min; 0% B at 0.35 mL/min for 0.5 min; linear gradient to 20% B at 15 min at 0.35 mL/min; linear gradient to 75% B at 55 min at 0.3 mL/min (flow rate constant for remainder of program); linear gradient to 90% B at 60 min; hold at 90% B until 85 min; linear gradient to 0% B at 90 min; hold at 0% B until 120 min. Spectra were acquired in positive ion mode with a scan range of 300–1500 m/z. We converted MS/MS data into peak list files using Extractmsn implemented in Bioworks (version 3.1; ThermoFinnigan) with the following parameters: peptide molecular weight range 300–3,500, threshold 100,000, precursor mass tolerance 1.4, minimum ion count 35. We conducted MS/MS database searches using Mascot

(licensed, version 2.2, Matrix Science, Boston, MA, USA) against the National Center for Biotechnology Information non-redundant (NCBInr) database within Chordata. A parent tolerance of 1.20 Da, fragment tolerance of 0.60 Da, and two missed trypsin cleavages were allowed. We specified carbamidomethylation of cysteine and oxidation of methionine in MASCOT as fixed and variable modifications, respectively.

Gel Filtration Chromatography

C. atrox venom for Cv-PC use was fractionated via size exclusion chromatography using a Superdex gel filtration column (HiLoad 16-/60 Superdex 75PG, 17-1068-02, GE Healthcare) and Amersham Biosciences ÄKTAFPLC (18-1900-26, GE Healthcare). Crude venom (4 mg/mL in 0.15 M ammonium bicarbonate) was injected into the column (500 mL sample) and separated in 0.15 M ammonium bicarbonate at a flowrate of 1 mL/min. Individual fractions were collected manually at the local minimums of each peak (based on absorbance at 214 nm). All fractions collected were lyophilized and stored at -20^{o}C until use. The concentrations of each reconstituted sample was measured using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific).

Minimal Stimulation In Vitro *Clotting/Platelet Testing*

Consenting normal adults donated 5 mL citrated blood (1 part citrate + 9 parts blood) for each agent to be tested. Blood was drawn into citrated syringes and mixed gently, then all air was expressed, a parafilm seal was placed over

luer tip. Syringes were maintained horizontally at 37˚C for approximately 50-60 min, allowing endothelial inhibitors to dissipate and platelet function to reach a stable phase. During this stable phase, multiple tests could be performed and the results compared.

Baseline platelet testing was done in duplicate at 37˚C as follows. Pre-warmed reaction tubes [containing 1 mg Celite 270 (Manville Products, Denver, CO 80217), 450 µL 0.0055M isotonic CaCl2 and 25-35 solid glass beads, 0.5mm (#030001, Propper Manuf Co, NY) were pre-mixed. To this 3 drops (~150 µL) of well-mixed blood were added and time to completion of the clumping, sticking and clotting visual endpoints were measured on the Hemostasis Mechanism Analyzer (Boehringer Laboratories, Norristown, PA). Endpoints were identified as follows: Clumping was noted when platelets adhered to each other and to the celite, forming visible clumps approximately the same size as the glass beads. Sticking was noted when platelet/celite clumps “stuck” to the walls of the cuvette and were carried around as the tube rotated. Clotting was identified when the majority of the beads were pulled towards the left side of the rotating tube and carried around out of sight.

When done with baseline testing and any necessary repeats, the blood level in each syringe was adjusted to exactly 3 mL in preparation for testing of the various venom concentrations. After remixing, 10 µL of the venom under test was carefully added midway in the column of 3 mL whole blood using a Drummond pipette. After exactly 30 seconds, the sample was remixed and testing was performed as described above. Testing was repeated periodically

over ~20 minutes to monitor changes in platelet and clotting function after exposure to the venom.

Statistical Analysis

All values are mean ± SEM. GraphPad Prism software was used for statistical analysis. As specified in each figure legend, 1-way ANOVA, with Tukey's comparisons, was used to determine differences among 3 or more. A *P* value <0.05 was considered significant.

Study Approval

All animal husbandry and procedures were approved by the Institutional Animal Care and Use Committee. The study protocol was approved by the Institutional Review Board at Loma Linda University.

Acknowledgements

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CHAPTER FIVE

CONCLUSIONS

Summary of Findings

Our studies examined the potential of Cv-PC in preventing SBI. Cv-PC reduced postoperative brain edema and improved neurological function in animals undergoing SBI. Cv-PC suppressed the over-expression of COX-2 following SBI. Evaluation of COX-2 inhibition during Cv-PC demonstrated reversal of Cv-PC beneficial effects on edema and neurological function.

Cv-PC was also observed to reduce both intraoperative hemorrhage and postoperative hematoma by over 30% in SBI animals. Cv-PC generated FSPs and increased plasma fibrinogen. While hemostasis was improved, Cv-PC did not cause clinically significant changes in PT, INR, PTT, D-Dimer, or soluble fibrin suggestive of a thrombotic state. MMP-inhibition abolished the improved coagulation by Cv-PC. Furthermore, venom fractions containing SVMPs maintained the ability to reduce hemorrhage by PC.

From our data, we can conclude that 1) Cv-PC reduces brain edema and neurological deficits by downregulating the PLA_2/COX-2 pathway; 2) Cv-PC increases plasma fibrinogen and decreases SBI-induced intraoperative hemorrhage and postoperative hematoma; 3) Cv-PC-induced increase in plasma fibrinogen is mediated by SVMPs; 4) Assessment of coagulation parameters suggest that Cv-PC does not cause a hypercoagulable, thrombotic state.

Clinical Implications

Relatively, little research has focused on understanding SBI's pathophysiology. The administration of nonspecific postoperative care has been the mainstay of treatment for neurosurgical patients (Bruder and Ravussin 1999, Hellwig, Bertalanffy et al. 2003). With the rising costs of healthcare, preventive measures grow increasingly relevant to medical practice**.** Even neurosurgical patients without life-threatening complications must be monitored closely in the critical care unit (Bruder and Ravussin 1999). The prevention of brain edema in neurosurgical patients would not only improve individual patient outcomes but could also shorten hospital stays, ultimately cutting perioperative costs. Reduction of perioperative hemorrhage has broader applications that extend beyond the field of neurosurgery. Over 200 million major elective surgeries are performed worldwide per year (Weiser, Regenbogen et al. 2008) and up to an estimated $6.03 million is spent annually per hospital on blood and transfusion-related care for surgical patients (Shander, Hofmann et al. 2010). Cv-PC has demonstrated its ability to reduce both edema and perioperative hemorrhage, making it a promising therapy to improve patient outcome and simplify perioperative care. Further studies are needed to isolated and characterize *Crotalus* venom components and to assess the translational viability of Cv-PC.

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