Published in final edited form as:

J Neurosci Res. 2020 January ; 98(1): 191–200. doi:10.1002/jnr.24334.

***Crotalus atrox* Disintegrin Reduces Hemorrhagic Transformation by Attenuating Matrix Metalloproteinase-9 Activity after Middle Cerebral Artery Occlusion in Hyperglycemic Male Rats**

Devin W. McBride^{1,2}, Eric C.K. Gren³, Wayne Kelln³, William K. Hayes³, John H. Zhang^{2,4,5}

¹The Vivian L. Smith Department of Neurosurgery, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, Texas, USA

²Department of Physiology & Pharmacology, Loma Linda University School of Medicine, Loma Linda, California, USA

³Department of Earth and Biological Sciences, Loma Linda University School of Medicine, Loma Linda, California, USA

⁴Department of Neurosurgery, Loma Linda University School of Medicine, Loma Linda, California, USA

⁵Department of Anesthesiology, Loma Linda University School of Medicine, Loma Linda, California, USA

Abstract

Hemorrhagic transformation after ischemic stroke is an independent predictor for poor outcome and is characterized by blood vessel rupture leading to brain edema. To date, no therapies for preventing hemorrhagic transformation exist. Disintegrins from the venom of *Crotalus atrox* have targets within the coagulation cascade, including receptors on platelets. We hypothesized that disintegrins from *Crotalus atrox* venom can attenuate hemorrhagic transformation by preventing activation of matrix metalloproteinase after middle cerebral artery occlusion in hyperglycemic rats. We subjected 48 male Sprague-Dawley rats weighing 240–260 g to middle cerebral artery occlusion and hyperglycemia to induce hemorrhagic transformation of the infarction. At reperfusion, we administered either saline (vehicle), whole *Crotalus atrox* venom (two different doses were used), or fractionated *Crotalus atrox* venom (HPLC Fraction 2) to animals at reperfusion. Rats were euthanized 24 hours post-ictus for measurement of infarction and hemoglobin volume. Reversed-phase HPLC was performed to fractionate the whole venom and peaks were combined to form Fraction 2, which contained the disintegrin Crotatroxin. Fraction 2

Address correspondence to: Devin W. McBride, Ph.D., The Vivian L. Smith Department of Neurosurgery, The University of Texas Health Science Center at Houston, Houston, Texas, USA 77030, Phone: (713) 500-6169, Fax: (713) 500-5590, devin.w.mcbride@uth.tmc.edu.

Author's Roles

Conceptualization: DWM and JHZ. Methodology: DWM, ECKG, and WK. Investigation: DWM, ECKG, and WK. Formal Analysis: DWM. Resources: WKH and JHZ. Writing: DWM. Writing - Revising: WKH and JHZ. Supervision and Funding Acquisition: JHZ.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Data Accessibility

All data is available provided a reasonable request is made.

protected against hemorrhagic transformation after middle cerebral artery occlusion, and attenuated activation of matrix metalloproteinase-9. Administering matrix metalloproteinase antagonists prevented the protection by Fraction 2. The results of this study indicate that disintegrins found in *Crotalus atrox* venom may have therapeutic potential for reducing hemorrhagic transformation after ischemic stroke. Moreover, the RP-HPLC fractions retained sufficient protein activity to suggest that gentler and less efficient orthogonal chromatographic methods may be unnecessary to isolate proteins and explore their function.

Graphical Abstract

Snake venom disintegrins may provide a novel therapeutic which can be administered after stroke to reduce the complications of hemorrhagic transformation. In rats subjected to middle cerebral artery occlusion, we show that this is feasible using a hemoglobin assay to assess post-euthanasia brain tissue.

Keywords

hemorrhagic transformation; MCAO; hyperglycemia; snake venom disintegrin; crotatroxin

Introduction

Ischemic stroke affects approximately 700,000 individuals per year in the United States (Benjamin et al., 2018), with 33–66% of stroke patients being hyperglycemic upon admission (Lindsberg & Roine, 2004). Hyperglycemia has long been recognized for its deleterious effects on outcome after ischemic stroke, leading to secondary injuries including brain edema and hemorrhagic transformation (Li et al., 2013). Specifically, hemorrhagic transformation within infarcted tissue is a critical factor in poor prognosis after stroke, and affects 13–43% of surviving stroke patients (Zhang, Yang, Sun, & Xing, 2014). There are currently no therapeutics used clinically to reduce the incidence of hemorrhagic transformation in stroke patients at high-risk for hemorrhagic transformation. A number of treatments are being explored in preclinical rodent models of stroke, but, to date, there is only one clinical trial investigating a drug for reducing the incidence of hemorrhagic

transformation post-stroke. Several experimental papers have highlighted matrix metalloproteinase-2 and -9 (MMP-2, MMP-9) as critical factors involved in promoting hemorrhagic transformation (Elgebaly et al., 2011; X. Liu, Ye, An, Pan, & Ji, 2013; Shi, Leak, Keep, & Chen, 2016).

The involvement of MMPs in BBB disruption, progression of infarction, and hemorrhagic transformation after ischemic stroke has been investigated in numerous studies. While there are eight MMPs which have functions in breaking down the BBB or contributing to larger infarctions (Arba et al., 2018; DeGracia, 2018; Lakhan, Kirchgessner, Tepper, & Leonard, 2013), MMP-2 and -9 are thought to be the primary targets for preventing MMP-induced BBB disruption and hemorrhagic transformation (Lakhan et al., 2013). MMP-2 is critically involved in the initial opening of the BBB following ischemic stroke (Reuter et al., 2015; Rosenberg et al., 1992; Suofu et al., 2012; Yang, Estrada, Thompson, Liu, & Rosenberg, 2007), whereas MMP-9 is the major factor involved in delayed opening of the BBB (Chen et al., 2017; H. Liu et al., 2016; Sang et al., 2017; Suofu et al., 2012). The latter findings have also been observed in patients (Castellanos et al., 2003; Gasche et al., 1999), suggesting that MMPs may be therapeutic targets (Ji et al., 2017).

Snake venoms offer a unique pool of proteins which interact with hematic processes through activation and/or inhibition of proteins (Marsh, 2001). The objective of this study was to determine the therapeutic value of venom from the western diamondback rattlesnake (*Cmtalus atrox*) for preventing hemorrhagic transformation after transient middle cerebral artery occlusion (MCAO) in hyperglycemic male rats. Our second objective was to identify a protein responsible for any observed therapeutic benefit for hyperglycemic rats following MCAO.

Materials and Methods

The experiments were approved by the Loma Linda University IACUC, conducted in compliance with the *NIH Guidelines for the Use of Animals in Neuroscience Research* and with the U.S. National Research Council's *Guide for the Care and Use of Laboratory Animals* and with the U.S. Public Health Service's *Policy on Humane Care and Use of Laboratory Animals*, and are reported in compliance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines.

Study Design and Data Handling

Randomization: we randomly assigned animals to the groups and endpoints without bias 24 hours before performing any surgical procedures. Blinding: all investigators responsible for functional assessment, measurement of outcomes, and data analysis were blinded to the experimental groups. Sample Size Estimation: we used a minimum detectable difference in means of 10 and a standard deviation of 4 (obtained from our previous publications on MCAO), a power of 0.80, and an alpha of 0.05 to determine that a total of 6 animals per group (5–6 surviving assuming a mortality of 1 in the injury groups) were required to test for statistical significance. Forty-eight adult male Sprague-Dawley rats (240–260 g) were used in all experiments. Animals were housed in a humidity-controlled and temperature-

controlled environment with a 12-hr light/dark cycle. Food and water were accessible at all times.

All animals used for this study were included. Animals expiring before the designed endpoints were not included in measurement and analysis of post-euthanasia endpoints (*i.e.* infarct volume, hemoglobin volume, MMP Zymography). No data were removed before analysis.

Animal Groups

Animal groups were sham (n=12), MCAO + Vehicle (n=12), MCAO + *C. atrox* (low dose (LD), n=6), MCAO + *C. atrox* (high dose (HD), n=6), MCAO + *C. atrox* Fraction 2 (n=6), and MCAO + (*C. atrox* Fraction 2 with batimastat and prinomastat) (n=6).

Middle Cerebral Artery Occlusion Model

Middle cerebral artery occlusion (MCAO) for 90 min or sham surgery was performed, as previously described (McBride, Klebe, Tang, & Zhang, 2015). Briefly, animals were anesthetized with ketamine (80 mg/kg) and xylazine (20 mg/kg, intraperitoneally), followed by atropine (0.1 mg/kg, subcutaneously). The right common, internal, and external carotid arteries were exposed, and the external carotid artery was ligated, leaving a 3–4 mm stump. The internal carotid artery was then located, and the pterygopalatine artery was ligated. Vascular clips were placed on the internal and common carotid arteries, and then the external carotid artery stump was re-opened. A 4.0 monofilament nylon suture with a rounded tip was advanced through the stump and into the internal carotid artery until resistance was felt. The suture remained in place for 90 min, after which the suture was removed, beginning reperfusion. Immediately after suture removal, animals were administered 100 μ L of either normal saline (vehicle), a low dose of *C. atrox* (0.184 mg/kg, LD), a high dose of *C. atrox* (0.276 mg/kg, HD) or *C. atrox* Fraction 2 (0.064 mg/kg) via tail vein injection. Animals were given buprenorphine (0.02 mg/kg, subcutaneously) and yohibime (1 mg/kg, intraperitoneally) and allowed to recover.

Two hours post-reperfusion, 50% dextrose (6 mL/kg) was injected intraperitoneally. Measurement of blood glucose was performed before MCAO, 1 hour post-dextrose injection (3 hours post-reperfusion, and 24 hours after reperfusion).

To antagonize the activity of disintegrins and matrix metalloproteinases (MMPs), batimastat (0.6 mg/kg, SML0041, Sigma-Aldrich) and prinomastat (0.6 mg/kg, PZ0198, Sigma-Aldrich) were incubated for 30 minutes at 37°C with *C. atrox* Fraction 2 (Howes, Theakston, & Laing, 2007; Ikeda et al., 1991). The mixture of venom and antagonists was stored at –20°C. One group of MCAO animals (n=6) was administered the mixture of *C. atrox* Fraction 2 with batimastat and prinomastat via tail vein injection immediately after reperfusion. Hyperglycemia was induced via intraperitoneal injection of 50% dextrose (6 mL/kg) 2 hours post-reperfusion.

Twenty-four hours after MCAO, animals were euthanized for measurement of infarction volume, hemoglobin volume, and MMP zymography. Briefly, animals were deeply anesthetized and transcardially perfused with PBS. Brains were quickly removed, sectioned

into 2 mm thick slices, stained with 2% triphenyl tetrazolium chloride (TTC, T8877, Sigma-Aldrich), and then imaged. Then the slices were separated into ipsilesional and contralesional hemispheres and snap-frozen.

Infarction and Brain Swelling

Images of the TTC-stained brain slices were used to determine the areas of the contralesional hemisphere (C_i), and the non-ischemic ipsilesional hemisphere (N_i) (ImageJ, NIH, RRID:SCR_003070). Infarct volume (percent of whole brain) (McBride et al., 2015) was calculated as

$$\text{Infarct Volume (\%)} = \left(\frac{\sum_i (C_i - N_i)}{2 \left(\sum_i C_i \right)} \right) 100$$

Hemoglobin Assay

Hemorrhagic transformation was quantified with the hemoglobin assay (Choudhri, Hoh, Solomon, Connolly, & Pinsky, 1997). Ipsilesional hemispheric brain tissue was added to 3 mL PBS then homogenized for 30 seconds and sonicated on ice for 1 minute. Samples were centrifuged (13,000 rpm, 4°C) twice for 30 minutes each. 0.2 mL of supernatant was added to 0.8 mL of Drabkin's reagent (Biorad) and incubated in the dark for 15 minutes at room temperature. The optical density at 540 nm was measured using a spectrophotometer (Genesys 10S, Thermoscientific). The hemoglobin volume of each sample was determined using a standard curve; error was propagated through all calculations. The standard curve was created by adding known amounts of blood to naïve hemispheric brain tissue and processed as described above. Linear regression of the absorbance versus hemoglobin volume was performed. Contralesional tissue was used as a control.

MMP-9 Zymography

Activity of MMP-9 was determined using Novex zymogram gels (ZY00100BOX, Life Technologies) following the manufacturer's protocol. Briefly, 50 ng of each ipsilesional hemisphere sample was loaded and electrophoresis was performed. Colloidal blue was used to stain the gels and gels were imaged. A molecular weight marker was loaded in the gels for identification of MMP-9 based on its molecular weight. Activity was normalized to the activity level of Sham within each gel.

Statistical analysis

All tests were two-sided and no further adjustment for multiple comparison was done for the overall number of tests. Data are presented as the individual data points, mean, and standard deviation (SD). Normality and equal variance were confirmed. All data except for blood glucose were analyzed using one-way ANOVAs with Tukey's *post-hoc* tests (GraphPad Prism 6, La Jolla, CA, USA, RRID:SCR_002798). Blood glucose data were analyzed using a repeated measures two-way ANOVA with Tukey *post-hoc*. A *p*-value of 0.05 was considered statistically significant.

Fractionation and Protein Identification of *C. atrox* Venom

Crotalus atrox venom (V7000, Sigma-Aldrich) was reconstituted in 0.065% TFA and 2% acetonitrile (Buffer A) to a concentration of 6 mg/mL. This solution was centrifuged for 10 min at 15,000 g to remove undissolved particles. The sample (100 µL) was fractionated using an AKTAmicro HPLC system (GE Healthcare Life Sciences, Piscataway, NJ, USA) fitted with two reversed-phase columns (SOURCE 5RPC ST polystyrene/divinyl benzene, 4.6×150 mm; GE Healthcare) run in series (flow rate=0.5 mL/min, linear gradient of 0–100% Buffer B (0.05% TFA, 80% acetonitrile) in 30 column volumes). Protein elution was monitored at 214 nm and the peaks were collected manually. Each peak was sent for LC-MS analysis. Chromatography fractionation was performed several more times and we combined several eluted peaks between 62 mL and 68 mL into what we term Fraction 2 (Figure 1). The combined peaks were dried in a speed-vac and then reconstituted in normal saline before injection into the animals.

LC-MS

Each analyzed fraction was reduced with dithiothreitol and alkylated with iodoacetamide (Kim et al., 2017). Proteins were digested with proteomics-grade porcine pancreatic trypsin (Sigma-Aldrich) and then desalted (C18 ZipTips, EMD Millipore, Billerica, MA, USA). The desalted tryptic peptides were analyzed with a ThermoFinnigan LCQ Deca XP spectrometer (ThermoFinnigan, Waltham, MA, USA) equipped with a PicoView 500 nanospray apparatus using Xcalibur software. Samples (20 µL) were separated on a C18 Biobasic bead column (New Objective, Woburn, MA, USA) with a mobile phase B of 98% acetonitrile, 2% water, and 0.1% formic acid. The program executed 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, RRID:SCR_014594) 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 (version 2.2, Matrix Science, Boston, MA, USA) against the National Center for Biotechnology Information non-redundant (NCBI nr) 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.

Results

No animals were excluded from this study. Mortality rates were 0% (0/12) for Sham, 25% (3/12) for MCAO + Vehicle, 8% (1/12) for MCAO + *C. atrox* (LD), 17% ($\frac{1}{6}$) for MCAO + *C. atrox* (HD), 17% ($\frac{1}{6}$) for MCAO + *C. atrox* Fraction 2, and 33% (2/6) for MCAO + (*C. atrox* Fraction 2 incubated with batimastat and prinomastat).

Blood Glucose

One hour post-injection of 50% dextrose, the blood glucose was significantly elevated above baseline values in all groups for the dose study (*i.e.* Sham, MCAO + Vehicle, MCAO + *C. atrox* (LD), MCAO + *C. atrox* (HD)) (Time Factor: $F_{2,36}=162.8$, $p<0.0001$; Group Factor: $F_{3,18}=1.318$, $p=0.2993$; Interaction: $F_{6,36}=0.8855$, $p=0.5155$; $n=5-6/\text{group}$). By 24 hours post-MCAO, blood glucose was back to similar levels as baseline in all animals (Figure 2).

For the venom fraction study (*i.e.* Sham, MCAO + Vehicle, MCAO + *C. atrox* Fraction 2, MCAO + (*C. C. atrox* fraction 2 incubated with batimastat and prinomastat)), identical statistical findings were observed with blood glucose elevated 1 hour after dextrose injection and returning to baseline values at 24 hours post-MCAO (Time Factor: $F_{2,34}=168.9$, $p<0.0001$; Group Factor: $F_{3,17}=0.0818$, $p=0.9690$; Interaction: $F_{6,34}=0.1217$, $p=0.9930$; $n=4-6/\text{group}$).

Infarct Volume and Hemoglobin Volume – Dose Study

Twenty-four hours after MCAO, surviving animals were euthanized for assessment of the infarction volume and hemorrhagic transformation using brain hemoglobin volume. All animals subjected to MCAO had significantly larger infarct volumes than sham animals. In treated MCAO animals, neither the low dose nor the high dose of *C. atrox* venom was able to reduce the infarct volume compared to MCAO + Vehicle ($F_{3,19}=15.15$, $p<0.0001$, $n=5-6/\text{group}$) (Figure 3a).

Rats receiving MCAO and treated with the vehicle presented with statistically higher brain hemoglobin volumes compared to sham animals ($p<0.0001$ Sham vs MCAO + Vehicle). MCAO animals receiving *C. atrox* had significantly smaller hemoglobin volumes compared to vehicle-treated rats ($p=0.0007$ MCAO + Vehicle vs MCAO + *C. atrox* (LD), $p=0.0017$ MCAO + Vehicle vs MCAO + *C. atrox* (HD)). No significant difference was observed for the hemoglobin volumes among the sham, MCAO + *C. atrox* (LD), and MCAO + *C. atrox* (HD) groups ($p=0.5075$ Sham vs MCAO + *C. atrox* (LD), $p=0.4289$ Sham vs MCAO + *C. atrox* (HD), $p=0.9970$ MCAO + *C. atrox* (LD) vs MCAO + *C. atrox* (HD)) ($F_{3,19}=14.03$, $p<0.0001$, $n=5-6/\text{group}$) (Figure 3b).

Venom Fractionation

Crude *C. atrox* venom was fractionated using HPLC and the protein components identified with LC-MS. Several HPLC peaks were combined to form Fraction 2 of the whole venom (red box in Figure 1). These peaks all contained isoforms of disintegrin Crotatroxin (Prot ID: VM2A_CROAT) as determined by mass spectrometry.

Infarct Volume and Hemoglobin Volume - *C. atrox* Fraction 2

After fractionation of the crude *C. atrox* venom, we tested the therapeutic benefit of the combined HPLC peaks comprising Fraction 2. *C. atrox* Fraction 2 did not significantly reduce infarct volume in hyperglycemic rats 24 hours post-MCAO compared to vehicle-treated animals ($p=0.0003$ Sham vs MCAO + *C. atrox* Fraction 2, $p=0.9875$ MCAO + Vehicle vs MCAO + *C. atrox* Fraction 2). Co-administration of two MMP inhibitors, batimastat and prinomastat, with *C. atrox* Fraction 2 did not significantly reduce the

infarction volume compared to MCAO + Vehicle and MCAO + *C. atrox* Fraction 2 ($p=0.0010$ Sham vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.9477$ MCAO + Vehicle vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.9947$ MCAO + *C. atrox* Fraction 2 vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat)) ($F_{3,15}=15.43$, $p<0.0001$, $n=4-6/\text{group}$) (**Figure 4a**).

Crotalus atrox Fraction 2 significantly attenuated hemoglobin volume within the brain 24 hours post-MCAO compared to vehicle-treated rats ($p=0.0003$ Sham vs MCAO + *C. atrox* Fraction 2, $p=0.0014$ MCAO + Vehicle vs MCAO + *C. atrox* Fraction 2). Administration of MMP inhibitors with *C. atrox* Fraction 2 reversed the beneficial effects of *C. atrox* Fraction 2 on hemoglobin volume post-MCAO ($p<0.0001$ Sham vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.4720$ MCAO + Vehicle vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.0290$ MCAO + *C. atrox* Fraction 2 vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat)) ($F_{3,15}=42.60$, $p<0.0001$, $n=4-6/\text{group}$) (**Figure 4b**).

Matrix Metalloproteinase-9 Activity

The results of MMP-9 zymography revealed that vehicle-treated rats subjected to hyperglycemic MCAO had significantly higher MMP-9 activity compared to sham animals ($p=0.0001$ Sham vs MCAO + Vehicle). When MCAO rats were treated with *C. atrox* Fraction 2, the activity of MMP-9 was attenuated as they became statistically indistinguishable from that of the sham group ($p=0.8997$ Sham vs MCAO + *C. atrox* Fraction 2, $p=0.0005$ MCAO + Vehicle vs MCAO + *C. atrox* Fraction 2). However, addition of MMP inhibitors to the *C. atrox* Fraction 2 reversed the beneficial effects of *C. atrox* Fraction 2 on MMP-9 activity ($p<0.0001$ Sham vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.9820$ MCAO + Vehicle vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat), $p=0.0003$ MCAO + *C. atrox* Fraction 2 vs MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat)) ($F_{3,15}=23.29$, $p<0.0001$, $n=4-6/\text{group}$) (**Figure 5**).

Discussion

One of the most debilitating complications following ischemic stroke is hemorrhagic transformation. Development of hemorrhagic transformation is known to cause massive brain edema, larger infarction, and poorer outcome (Li et al., 2013). Several mechanisms may be responsible for hemorrhagic transformation, one of which is hyperglycemia. In this study, we sought to identify a protein in the venom of *C. atrox* which may be useful in reducing the incidence and burden of hemorrhagic transformation.

Herein, hyperglycemic rats subjected to MCAO were treated with *C. atrox* venom. We observed a marked decrease in the amount of hemoglobin in the brain tissue after MCAO, despite no change in the volume of infarction. We then fractionated the whole venom using reversed-phase HPLC and identified the disintegrin Crotatroxin as a potential candidate for reducing hemorrhagic transformation. The disintegrins and metalloproteinases contained within the venom of pit vipers (such as *Crotalus*) have been documented to influence coagulation, platelet activation/aggregation, and interaction with endothelial cells and extracellular matrix components (Bajwa, Markland, & Russell, 1981; Marsh, 2001; Schaeffer, Briston, Chilton, & Carlson, 1984). Post-HPLC fractionation, we combined

several peaks within the HPLC chromatogram to form *C. atrox* Fraction 2 which consisted of disintegrin Crotatroxin. This fraction was then used to treat hyperglycemic MCAO rats. *Crotalus atrox* Fraction 2 provided a reduction of the hemoglobin volume within the brain tissue. Furthermore, incubation of two pan-MMP antagonists with *C. atrox* Fraction 2 blocked the beneficial effects of Fraction 2 on hemoglobin extravasation. Finally, in rats treated with *C. atrox* Fraction 2, the activity of MMP-9 was significantly reduced compared to MCAO rats treated with the vehicle.

Venoms offer a large unique pool of proteins that have been selectively adapted for interaction with other species. Snake venom proteins are typically classified as non-enzymatic proteins/peptides, neurotoxins, cardiotoxins, and hemotoxins (e.g. disintegrins and metalloproteinases) (Chan et al., 2016; Lee, 1979). Hemotoxic venom proteins, which exert various effects on blood coagulation, blood cells, and the vascular lining (Bajwa et al., 1981; Marsh, 2001; Schaeffer et al., 1984), are particularly relevant to this study. Interestingly, some snake venoms contain both pro-coagulant and anti-coagulant proteins. We previously demonstrated that whole *C. atrox* venom reduces perioperative hemorrhage in a rat model of surgical brain injury (Kim et al., 2017). Therefore, we hypothesized that there would be a protein(s) which could be harnessed for therapeutic benefit to protect against hemorrhagic transformation. We isolated the disintegrin Crotatroxin using HPLC fractionation and LC-MS, and tested it in a rat model of hemorrhagic transformation. Additional investigations are needed to determine the mechanism by which the disintegrin Crotatroxin confers its protection.

An interesting observation is the fact that the MMP-9 activity was reversed when Fraction 2 is incubated with MMP antagonists. This is likely because the MMP antagonists were not at a working concentration within the blood of the rat; the MMP antagonists were mixed with Fraction 2 and the mixture incubated prior to administering to the rat. The dose of MMP antagonist in the Fraction 2 solution was adequate for inhibiting the MMPs within the fraction. However, this dose was not high enough to provide systemic/CNS inhibition of endogenous MMPs. Thus, the MMP zymography results show a higher MMP activity for the fraction 2 + MMP antagonists. What is being observed is an inhibition of the fraction 2 disintegrins which allows for endogenous MMP activity to remain high after MCAO.

Of note, reversed-phase chromatography is generally assumed to denature proteins, and orthogonal chromatography methods are therefore typically employed when protein activity is to be retained. However, we demonstrated robust protein activity following RP-HPLC, suggesting that gentler orthogonal chromatographic protocols, which are time consuming, costly, and tend to dramatically reduce final protein yield, may not always be warranted.

Limitations and Future Studies

The current study has several limitations which need to be addressed in future studies. First, this study was exploratory in nature and only pseudo-mechanistic. *Cmtalus atrox* contains a multitude of proteins and peptides performing various mechanisms of action. We observed a reduction in brain hemoglobin volume for MCAO rats treated with whole venom, so we sought to identify a responsible protein. The protein identified herein was a disintegrin which may have effects on blood coagulation and platelets, the vasculature, and extracellular

matrix, as well as MMPs. Therefore, we utilized two pan-MMP antagonists to inhibit the activity of disintegrin to confirm that the disintegrin was responsible for the observed protection. Additional studies are required to determine the mechanism of action. Second, other proteins may also be responsible for the protection by the whole, crude venom. While *C. atrox* Fraction 2 partially attenuated the hemoglobin volume, treatment with the whole venom reduced hemoglobin volume back to levels similar to that of sham. Third, hyperglycemia was induced after 2 hours after reperfusion (*i.e.* 2 hours after drug administration). In preliminary experiments, we found that hemorrhagic transformation occurred too rapidly for the venom to prevent/attenuate it ((McBride et al., 2016) and unpublished data). Thus, it remains unclear whether whole *C. atrox* (and Fraction 2) preconditioned against hemorrhagic transformation or whether it was a treatment/pre-treatment. Finally, this study utilized only young adult male rats. Following the guidelines from STAIR (“Recommendations for standards regarding preclinical neuroprotective and restorative drug development,” 1999) and STEPS (“Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke,” 2009), this study should be repeated using female and aged rats, as well as investigating other comorbidities.

Conclusion

Herein we report that *C. atrox* venom is capable of attenuating hemorrhagic transformation after MCAO in hyperglycemic adult male rats. Furthermore, we found that Crotoxin, a disintegrin in *C. atrox* venom, is at least partially responsible for the observed therapeutic benefit provided by the whole, crude venom. Additional studies are warranted to identify other potentially therapeutic proteins from *C. atrox* venom.

Acknowledgements

Funding support was provided by a seed grant provided by The Vivian L. Smith Department of Neurosurgery at The University of Texas Health Science Center at Houston (DWM) and a NIH R01 NS084921 grant (JHZ).

Literature Cited

- Arba F, Piccardi B, Palumbo V, Giusti B, Nencini P, Gori AM, . . . Inzitari D (2018). Small Vessel Disease Is Associated with Tissue Inhibitor of Matrix Metalloproteinase-4 After Ischaemic Stroke. *Transl Stroke Res*. doi:10.1007/s12975-018-0627-x
- Bajwa SS, Markland FS, & Russell FE (1981). Fibrinolytic and fibrinogen clotting enzymes present in the venoms of western diamondback rattlesnake, *Crotalus atrox*, eastern diamondback rattlesnake, *Crotalus adamanteus*, and southern Pacific rattlesnake, *Crotalus viridis helleri*. *Toxicon*, 19(1), 53–59. [PubMed: 7222089]
- Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, . . . Muntner P (2018). Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation*, 137(12), e67–e492. doi:10.1161/cir.0000000000000558 [PubMed: 29386200]
- Castellanos M, Leira R, Serena J, Pumar JM, Lizasoain I, Castillo J, & Davalos A (2003). Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. *Stroke*, 34(1), 40–46.
- Chan YS, Cheung RC, Xia L, Wong JH, Ng TB, & Chan WY (2016). Snake venom toxins: toxicity and medicinal applications. *Appl Microbiol Biotechnol*, 100(14), 6165–6181. doi: 10.1007/s00253-016-7610-9 [PubMed: 27245678]

- Chen H, Guan B, Chen X, Chen X, Li C, Qiu J, . . . Shen J (2017). Baicalin Attenuates Blood-Brain Barrier Disruption and Hemorrhagic Transformation and Improves Neurological Outcome in Ischemic Stroke Rats with Delayed t-PA Treatment: Involvement of ONOO(-)-MMP-9 Pathway. *Transl Stroke Res*. doi:10.1007/s12975-017-0598-3
- Choudhri TF, Hoh BL, Solomon RA, Connolly ES Jr., & Pinsky DJ (1997). Use of a spectrophotometric hemoglobin assay to objectively quantify intracerebral hemorrhage in mice. *Stroke*, 28(11), 2296–2302. [PubMed: 9368579]
- DeGracia DJ (2018). Disease of mRNA Regulation: Relevance for Ischemic Brain Injury. *Transl Stroke Res*, 9(3), 251–257. doi:10.1007/s12975-017-0586-7 [PubMed: 29127592]
- Elgebaly MM, Oghi S, Li W, Mezzetti EM, Prakash R, Johnson MH, . . . Ergul A (2011). Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke. *Transl Stroke Res*, 2(3), 391–398. doi:10.1007/s12975-011-0083-3 [PubMed: 21909340]
- Gasche Y, Fujimura M, Morita-Fujimura Y, Copin JC, Kawase M, Massengale J, & Chan PH (1999). Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood-brain barrier dysfunction. *J Cereb Blood Flow Metab*, 19(9), 1020–1028. doi:10.1097/00004647-199909000-00010 [PubMed: 10478654]
- Howes JM, Theakston RD, & Laing GD (2007). Neutralization of the haemorrhagic activities of viperine snake venoms and venom metalloproteinases using synthetic peptide inhibitors and chelators. *Toxicon*, 49(5), 734–739. doi:10.1016/j.toxicon.2006.11.020 [PubMed: 17196631]
- Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, . . . et al. (1991). The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*, 87(4), 1234–1240. doi:10.1172/jci115124 [PubMed: 2010539]
- Ji B, Zhou F, Han L, Yang J, Fan H, Li S, . . . Xu Y (2017). Sodium Tanshinone IIA Sulfonate Enhances Effectiveness Rt-PA Treatment in Acute Ischemic Stroke Patients Associated with Ameliorating Blood-Brain Barrier Damage. *Transl Stroke Res*, 8(4), 334–340. doi:10.1007/s12975-017-0526-6 [PubMed: 28243834]
- Kim CH, McBride DW, Raval R, Sherchan P, Hay KL, Gren EC, . . . Zhang JH (2017). Crotalus atrox venom preconditioning increases plasma fibrinogen and reduces perioperative hemorrhage in a rat model of surgical brain injury. *Sci Rep*, 7, 40821. doi: 10.1038/srep40821 [PubMed: 28102287]
- Lakhan SE, Kirchgessner A, Tepper D, & Leonard A (2013). Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. *Front Neurol*, 4, 32. doi:10.3389/fneur.2013.00032 [PubMed: 23565108]
- Lee C-Y (Ed.) (1979). *Snake Venoms* (Vol. 52): Springer-Verlag Berlin Heidelberg.
- Li WA, Moore-Langston S, Chakraborty T, Rafols JA, Conti AC, & Ding Y (2013). Hyperglycemia in stroke and possible treatments. *Neurol Res*, 35(5), 479–491. doi:10.1179/1743132813y.0000000209 [PubMed: 23622737]
- Lindsberg PJ, & Roine RO (2004). Hyperglycemia in acute stroke. *Stroke*, 35(2), 363–364. doi: 10.1161/01.str.0000115297.92132.84 [PubMed: 14757880]
- Liu H, Wang Y, Xiao Y, Hua Z, Cheng J, & Jia J (2016). Hydrogen Sulfide Attenuates Tissue Plasminogen Activator-Induced Cerebral Hemorrhage Following Experimental Stroke. *Transl Stroke Res*, 7(3), 209–219. doi:10.1007/s12975-016-0459-5 [PubMed: 27018013]
- Liu X, Ye M, An C, Pan L, & Ji L (2013). The effect of cationic albumin-conjugated PEGylated tanshinone IIA nanoparticles on neuronal signal pathways and neuroprotection in cerebral ischemia. *Biomaterials*, 34(28), 6893–6905. doi:10.1016/j.biomaterials.2013.05.021 [PubMed: 23768781]
- Marsh NA (2001). Diagnostic uses of snake venom. *Haemostasis*, 31(3–6), 211–217. doi:48065 [PubMed: 11910187]
- McBride DW, Klebe D, Tang J, & Zhang JH (2015). Correcting for Brain Swelling's Effects on Infarct Volume Calculation After Middle Cerebral Artery Occlusion in Rats. *Transl Stroke Res*, 6(4), 323–338. doi:10.1007/s12975-015-0400-3 [PubMed: 25933988]
- McBride DW, Legrand J, Krafft PR, Flores J, Klebe D, Tang J, & Zhang JH (2016). Acute Hyperglycemia Is Associated with Immediate Brain Swelling and Hemorrhagic Transformation After Middle Cerebral Artery Occlusion in Rats. *Acta Neurochir Suppl*, 121, 237–241. doi: 10.1007/978-3-319-18497-5_42 [PubMed: 26463955]

- Recommendations for standards regarding preclinical neuroprotective and restorative drug development. (1999). *Stroke*, 30. doi:10.1161/01.str.30.12.2752
- Reuter B, Rodemer C, Grudzenski S, Meairs S, Bugert P, Hennerici MG, & Fatar M (2015). Effect of simvastatin on MMPs and TIMPs in human brain endothelial cells and experimental stroke. *Transl Stroke Res*, 6(2), 156–159. doi:10.1007/s12975-014-0381-7 [PubMed: 25476155]
- Rosenberg GA, Kornfeld M, Estrada E, Kelley RO, Liotta LA, & Stetler-Stevenson WG (1992). TIMP-2 reduces proteolytic opening of blood-brain barrier by type IV collagenase. *Brain Res*, 576(2), 203–207. [PubMed: 1381261]
- Sang H, Qiu Z, Cai J, Lan W, Yu L, Zhang H, . . . Zhang R (2017). Early Increased Bradykinin 1 Receptor Contributes to Hemorrhagic Transformation After Ischemic Stroke in Type 1 Diabetic Rats. *Transl Stroke Res*, 8(6), 597–611. doi:10.1007/s12975-017-0552-4 [PubMed: 28726167]
- Schaeffer RC Jr., Briston C, Chilton SM, & Carlson RW (1984). Hypotensive and hemostatic properties of rattlesnake (*Crotalus viridis helleri*) venom and venom fractions in dogs. *J Pharmacol Exp Ther*, 230(2), 393–398. [PubMed: 6747842]
- Shi Y, Leak RK, Keep RF, & Chen J (2016). Translational Stroke Research on Blood-Brain Barrier Damage: Challenges, Perspectives, and Goals. *Transl Stroke Res*, 7(2), 89–92. doi: 10.1007/s12975-016-0447-9 [PubMed: 26757714]
- Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. (2009). *Stroke*, 40(2), 510–515. doi: 10.1161/strokeaha.108.526863 [PubMed: 19095993]
- Suofu Y, Clark JF, Broderick JP, Kurosawa Y, Wagner KR, & Lu A (2012). Matrix metalloproteinase-2 or -9 deletions protect against hemorrhagic transformation during early stage of cerebral ischemia and reperfusion. *Neuroscience*, 212, 180–189. [PubMed: 22521821]
- Yang Y, Estrada EY, Thompson JF, Liu W, & Rosenberg GA (2007). Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J Cereb Blood Flow Metab*, 27(4), 697–709. doi:10.1038/sj.jcbfm.9600375 [PubMed: 16850029]
- Zhang J, Yang Y, Sun H, & Xing Y (2014). Hemorrhagic transformation after cerebral infarction: current concepts and challenges. *Ann Transl Med*, 2(8), 81. doi:10.3978/j.issn.2305-5839.2014.08.08 [PubMed: 25333056]

Significance Statement

Hemorrhagic transformation after ischemic stroke is a major deleterious event that is responsible for poor outcome. A factor causing hemorrhagic transformation is hyperglycemia. To date, there are no therapeutics available for preventing hemorrhagic transformation. We utilized *C. atrox* venom to identify a disintegrin which can attenuate hemorrhagic transformation following ischemic stroke in hyperglycemic rats.

Figure 1. HPLC chromatograph of crude venom from *C. atrox*. The red box indicates the peaks combined to form Fraction 2 used in this study. These peaks contained isoforms of disintegrin Crotatroxin (Prot ID: VM2A_CROAT).

Figure 2.

Dextrose Administered 2 Hours post-Reperfusion Induces Hyperglycemia. All animals had blood glucose (mg/dL) testing before MCAO (Baseline), 3 hours after reperfusion which is also 1 hour after dextrose injection (1 Hr Post-Dextrose Injection), and before sacrifice (24 Hrs Post-MCAO). One cohort of Sham and MCAO + Vehicle, and MCAO + *C. atrox* (LD) and MCAO + *C. atrox* (HD) was analyzed for statistical significance (n=5-6/group). Another cohort of Sham and MCAO + Vehicle, and MCAO + *C. atrox* Fraction 2 and MCAO + (*C. atrox* Fraction 2 + Batimastat + Prinomastat) was analyzed for statistical significance (n=4-6/group). All interventions were administered immediately after MCAO. Analyzed using two-way ANOVAs with Tukey *post-hoc* tests. * $p < 0.05$ vs Baseline, # $p < 0.05$ vs 1 Hr Post-Dextrose Injection.

Figure 3.

Crotalus atrox Venom Reduces Hemoglobin Volume, but Not Infarction Volume 24 hours after MCAO. (a) Representative images of TTC-stained brains. (b) Infarction volume (%) is significantly larger in MCAO rats compared to sham animals. Neither low nor high dose *C. atrox* venom reduced infarct volume. (c) Brain hemoglobin volume was significantly higher in MCAO rats treated with the vehicle compared to sham rats. *C. atrox* venom treatment significantly attenuates hemoglobin volumes in the brain. All interventions were administered immediately after MCAO. n=5-6/group. Analyzed using one-way ANOVAs with Tukey *post-hoc* tests. * $p < 0.05$ vs Sham, # $p < 0.05$ vs MCAO + Vehicle.

Figure 4.

Crotalus atrox Disintegrin Crotatroxin (Fraction 2) Attenuates Hemoglobin Volume, but Not Infarction Volume 24 hours after MCAO. (a) Representative images of TTC-stained brains. (b) MCAO causes significantly larger infarct volumes compared to sham surgery. *C. atrox* Fraction 2 has no effect on infarct volume compared to vehicle-treated animals. Co-administration of MMP inhibitors with *C. atrox* Fraction 2 similarly does not have any effect on infarction. (c) MCAO rats receiving vehicle treatment had statistically more brain hemoglobin volume compared to sham rats. *C. atrox* Fraction 2 significantly reduces hemoglobin volumes in the brain. Co-administration of MMP inhibitors reverses the protective effect of *C. atrox* Fraction 2 on hemoglobin volume. All interventions were administered immediately after MCAO. n=4-6/group. Analyzed using one-way ANOVAs with Tukey *post-hoc* tests. * $p < 0.05$ vs Sham, # $p < 0.05$ vs MCAO + Vehicle, & $p < 0.05$ vs MCAO + *C. atrox* Fraction 2.

Figure 5.

Crotalus atrox Disintegrin Crotatroxin (Fraction 2) Attenuates MMP-9 Activity 24 Hours Post-MCAO. MMP-9 zymography of rat brain shows that animals treated with the vehicle had significantly greater MMP-9 activity compared to sham rats. The MMP-9 activity was reduced back to sham levels in rats receiving *C. atrox* Fraction 2. The protective effects of *C. atrox* Fraction 2 on MMP-9 activity was reversed by administration of MMP inhibitors. All interventions were administered immediately after MCAO. n=4-6/group. Analyzed using one-way ANOVA with Tukey *post-hoc* test. * $p < 0.05$ vs Sham, # $p < 0.05$ vs MCAO + Vehicle, & $p < 0.05$ vs MCAO + *C. atrox* Fraction 2.