

**RESEARCH REPORT****Capillary damage in the area postrema by venom of the northern black-tailed rattlesnake (*Crotalus molossus molossus*)**

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**ABSTRACT**

The Northern black-tailed rattlesnake (*Crotalus molossus molossus*) venom is mainly hemotoxic, hemorrhagic, and neurotoxic. Its effects in the central nervous system are unknown and only poorly described for all Viperidae species in general. This is why we are interested in describe the damage induced by *C. m. molossus* venom in rat brain, particularly in the area postrema capillaries. Four *C. m. molossus* venom doses were tested (0.02, 0.05, 0.10 and 0.20mg/kg) injected intramuscularly at the lower limb, incubated by 24 hours and the brains were harvested. Area postrema coronal sections were stained with Haematoxylin and Eosin, and examined to observe the venom effect in quantity of capillaries and porphology. Starting from the 0.10mg/kg treatment we observed lysed extravasated erythrocytes and also capillary breakdown, as a consequence of hemorrhages appearance. The number of capillaries decreased significantly in response to the venom dose increment. Hemorrhages could be caused by the metalloproteinase activity on the basal membrane and the apoptosis generated by L-amino acid oxidases. Hemolysis could be caused by phospholipase A<sub>2</sub> hemotoxic effect. We conclude that *C. m. molossus* crude venom produces hemolysis, capillary breakdown, hemorrhages, and the reduction in number of capillaries in the area postrema.

**KEYWORDS:** *Crotalus molossus molossus*, area postrema, metalloproteinase, L-amino acid oxidase, phospholipase A<sub>2</sub>

**INTRODUCTION**

In Mexico, 27,480 cases of ophidic accidents were reported in 2007, in which 0.5% of the cases ends in death (Luna-Bauza, 2007). Although mortality due to an ophidic accident is not a major problem, in many cases it can result in functional disability, loss of extremities, and costly recovery (Sánchez et al, 2003). *C. m. molossus* is distributed in the southwestern United States in Arizona, New Mexico, and Texas, and northern Mexico in Sonora, Chihuahua, and Coa-

huila (Lemos-Espinal and Smith, 2007). *Crotalus* venom contains a complex mixture of proteins with and without enzymatic activity, the most representative are the P-I and P-III metalloproteinases, serine proteinases, L-amino acid oxidases, phospholipase A<sub>2</sub>, disintegrin, cysteine-rich secretory protein, and others (Mackessy, 2010). *C. m. molossus* venom has a phosphodiesterase (Ferlan et al, 1983a) two metalloproteinases (Rael et al, 1992; Chen and Rael, 1997; Sánchez et al, 2001), a disintegrin called molossin (Scarborough et al, 1993), and three phospholipase A<sub>2</sub> (Ferlan et al,

1983b; Tsai et al, 2001), that had been isolated and characterized. Together all these proteins cause platelet aggregation (Hardy et al, 1982; Corrigan et al, 1983), hemorrhages, proteolysis (Soto et al, 1989), and fibrinolysis (Perez et al, 2001).

The neurological effect of the crotalids venom on the central nervous system has not been well described, but there are some medical cases were ischemic stroke (Thomas et al, 2006), intracranial hemorrhages, and cerebral infarctions (Del Brutto and Del Brutto, 2012; Rebahi et al, 2014) are reported. Experimentally, it has been shown that *Bothrops colombiensis* venom can cause erythrocyte extravasation at leptomeninges (Rodríguez-Acosta et al, 2003), and *Hypnale zara* venom can cause ischemic neuronal degeneration in cerebral cortex (Silva et al, 2012). Moreover, experimentation with specific venom toxins reveals damage in the brain, for example, phospholipase A2 from *Echis carinatus* generates vacuole formation in the cytoplasm of prefrontal cortex cells (Perumal Samy et al, 2010), and gyroxin, a *Crotalus durissus terrificus* serine protease, can cause histological changes in cerebellum and prefrontal cortex (Ruiz de Torrent et al, 2007) and, temporally, is able to disturb blood brain barrier permeability (Alves da Silva et al, 2011). These evidences suggest that the venom components could pass through blood-brain barrier and cause this effect.

Area postrema is a brain structure that lacks of blood-brain barrier, is highly vascularized and contains fenestrated capillaries without tight junctions between endothelial cells, through which molecules may pass freely from the circulation into the central nervous system (Cottrell and Ferguson, 2004; Maalood and Meister, 2009). It is chemosensitive to toxins in blood, also controls respiratory and renal functions, among others (Willis et al, 2007). Thus, we described the damage in the area postrema capillaries of rat induced by the black-tailed rattlesnake *C. m. molossus* crude venom.

## MATERIAL AND METHODS

### Materials

Folin-Ciocalteu's phenol reagent, bovine serum albumin, acrylamide, bis-acrylamide, TEMED, Coomassie brilliant blue R-250, paraformaldehyde, Sodium hydroxide, sodium borate decahydrate, haematoxylin and eosin Y were from Sigma (St. Louis, MO). Sodium carbonate, sodium tartrate, cupric sulphate decahydrate were from J. T. Baker (Center Valley, PA). Sodium dodecyl sulfate, Tris-HCl, ammonium persulfate were from Gibco BRL (Grand Island, NY). Tissue-Tek® OCT was from Sakura Finetek (Torrance, CA)

### Venom

Venom was extracted from two female adult *C. m. molossus* specimens maintained at the Laboratorio de Ecología y Biodiversidad Animal of Universidad Autónoma de Ciudad Juárez. The snakes were allowed to bite into a paraffin membrane over a beaker; the venom was pooled, transferred to 1.5ml microtube, and stored at -20°C. For experiments, the venom was used within four months of the collection. Protein concentration of venom was determined by the method

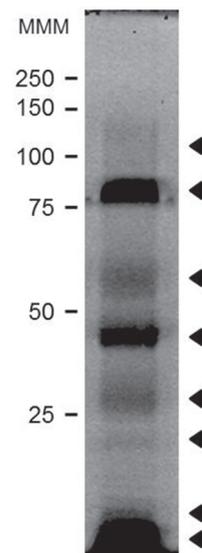
of Lowry et al (1951). Venom proteins were visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 12% (w/v) polyacrylamide slab gel following the method of Laemmli (1970). Protein bands were observed by Coomassie Brilliant Blue R-250 staining procedure.

### Animals

The animals used in this study were 15 female Sprague-Dawley rats (150–180gm body weight) of three month old obtained from animal housing facility of Universidad Autónoma de Ciudad Juárez and maintained at room temperature with food and water *ad libitum*. Ethical clearance for the study was obtained from the Ethics review committee of the Instituto de Ciencias Biomédicas of Universidad Autónoma de Ciudad Juárez.

### Treatments and histopathological examination

Five treatments of three rats were tested, all rats were intramuscularly injected in the lower limb with 100µl of 0.00 (as a control), 0.02, 0.05, 0.10, and 0.20mg/kg of venom dissolved in physiological saline solution. After 24hrs, the animals were sacrificed with an intraperitoneal pentobarbital sodium injection (380mg/kg). The brains were quickly harvested, fixed in paraformaldehyde (4%, v/v, paraformaldehyde, 0.1M sodium borate decahydrate, 0.4%, w/v, sodium hydroxide, pH 9.5) for 24hrs, dehydrated with 30% (w/v) sucrose for 24hrs. The brains were frozen in Tissue-Tek® OCT and cut (10µm) in a cryostat Leica CM1510 S. Three coronal sections of area postrema from each brain were obtained at -13.80 to -14.04mm bregma coordinates (Paxinos and Watson, 2005). Sections were stained with Harris' Haematoxylin and Eosin (Allen, 1995), and examined with a light microscope Leica DM 2000.



**Figure 1.** SDS-PAGE of *C. m. molossus* venom. All toxins are indicated on the right side of the gel (arrow heads) and molecular mass marker (MMM) on the left. Samples were separated using 12% (w/v) polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

### Capillary quantification

Capillaries were quantified from each area postrema section, using three sections per rat and three rats per treatment, considering approximately a 0.18mm<sup>2</sup> surface of the structure. Means of capillaries per treatment were calculated and ANOVA test ( $P < 0.05$ ) was applied.

## RESULTS

### Venom

In the SDS-PAGE 8 bands were visualized of 113.2, 94.5, 55.1, 40.4, 26.2, 20.2, 13.69 and 11.0kDa (Figure 1).

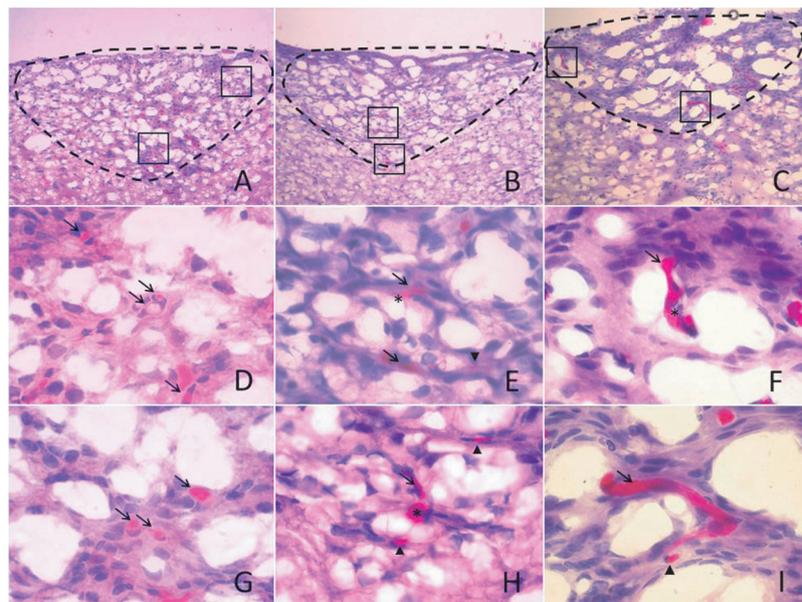
### Area postrema

The area postrema showed no histological damage in control, 0.02, and 0.05mg/kg treatments. In the 0.10 and 0.20mg/kg treatments, extravasated erythrocytes and capillary breakdown was induced, and as consequence hemorrhages appeared in both treatments (Figure 2). Hemolysis was observed in the 0.10 and 0.20mg/kg treatments (Figure 3) at peripheral zones of the area postrema such as central canal and the space between the spinal cord and the cerebellum.

Figure 4 shows the effect of the venom in all capillaries counted per area postrema per treatment. The control treatment showed the maximum quantity of capillaries of all treatments and it significantly decreased ( $P < 0.05$ ) in response to the venom dose increase, being the 0.20mg/kg dose the treatment with the lower quantity of capillaries. Between 0.02 and 0.05mg/kg treatments no significant difference was observed, as occurred among 0.10 and 0.20mg/kg treatments.

## DISCUSSION

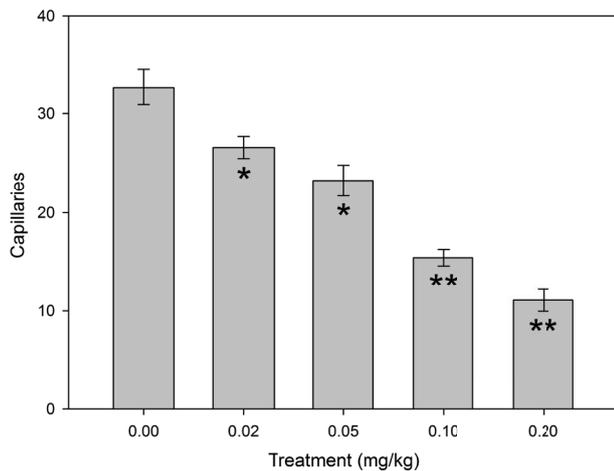
Following the results of Mackessy (2010) all the bands of our *C. m. molossus* venom sample were identified: the 11.0kDa band corresponds to a disintegrin (Scaborough et al, 1993) or a phospholipase A2 (Tsai et al, 2001); the 11.6 and 13.7 are different isomers of phospholipase A2; the 26.2kDa to a PI metalloproteinase (Chen and Rael, 1997; Sánchez et al, 2001); the 40.4kDa to a serine proteinase; the 55.1kDa band to a PIII metalloproteinase; the 94.5kDa band to a L-amino acid oxidase and the 113.13kDa to a nuclease or a phosphodiesterase (Ferlan et al, 1983a).



**Figure 2.** Venom effect in capillaries of the area postrema. Haematoxylin and Eosin staining of 10µm area postrema coronal sections. Control (A, D and G), 0.10mg/kg treatment (B, E, H) and 0.20mg/kg treatment (C, F and I). Area postrema in A, B and C are delimited by a discontinuous line, boxes in A, B and C indicate higher magnifications as shown in D, G; E, H and F, I, respectively. Control show intact capillaries (arrow) only, 0.10 and 0.20mg/kg treatments show capillaries with hemorrhages (asterisk) and extravasated erythrocytes (arrow head). A, B and C were observed in 20× magnification, D, E, F, G, H and I were observed in 100× magnification.



**Figure 3.** Hemolysis caused by *C. m. molossus* venom. Undamaged erythrocyte (A) from control, damaged erythrocytes are shown on 0.10 (B) and 0.20 (C, D and E) mg/kg treatments.



**Figure 4.** Effect of the *C. m. molossus* venom on the quantity of the area postrema capillaries. Bars are mean  $\pm$  S.E.M. of area postrema capillaries from three coronal sections ( $n=3$  per treatment). 0.02 and 0.05 mg/kg treatments showed no significant difference (\*) between them, neither between 0.10 and 0.20 mg/kg treatments (\*\*) ( $P < 0.05$ ).

The capillary damage in the area postrema was progressive in response to the increase of venom dose. The capillary breakdown and hemorrhages showed in 0.10 and 0.20 mg/kg treatments could be caused, firstly, by the degradation of basal membrane structural proteins as fibrin, nidogen, laminin and IV collagen by the P-I and P-III metalloproteinases (Sánchez et al, 2001; Escalante et al, 2006; Baldo et al, 2010). These enzymes can generate endothelial cells apoptosis (Díaz et al, 2005; Tanjoni et al, 2005). Secondly, hydrogen peroxide production by the activity of the L-amino acid oxidase could cause a cytotoxic environment for the endothelial cells ending in apoptosis (Guo et al, 2012).

Hemolysis found in the 0.10 and 0.20 mg/kg treatments could be caused by phospholipase A2 activity over erythrocyte cell membrane phospholipids (Du et al, 1998). Furthermore, L-amino acid oxidase contributes to hemolysis (Ali et al, 2000); particularly, *C. m. molossus* venom has been reported as a cause of this effect in vitro (Macias-Rodriguez et al, 2014).

The quantitative analysis of area postrema capillaries demonstrates a significant decrease of the number of capillaries as a consequence of the increment of the *C. m. molossus* venom dose. This event could be originated by the serine proteinase activity over the blood coagulation pathway (Pérez et al, 2007) and P-III metalloproteinase can promote prothrombin activation (Fox and Serrano, 2010). Suggesting that the ischemic stroke described in some medical cases (Thomas et al, 2006; Del Brutto and Del Brutto, 2012) could be caused by this phenomena, not only in the area postrema but in the whole central nervous system. Even though neurotoxic affections are not observed in envenomation by *C. m. molossus* (Hardy et al, 1982; Yarema and Curry, 2005) probably because only three clinical cases have been reported, but several cerebrovascular complications have been reported by crotalid envenomation (Thomas et al, 2006; Cardoso-Vale et al, 2013; Del Brutto and Del Brutto, 2012).

Morphological damage in the area postrema could generate a physiological alteration probably does not result in a renal failure or a respiratory paralysis, but maybe to predispose these symptoms of the envenomation by *Crotalus* sp venom (Sarmiento-Acuña, 2012).

## CONCLUSIONS

*C. m. molossus* crude venom causes hemolysis, capillary breakdown, hemorrhages, and reduction in number of capillaries in the area postrema of rats starting from a low venom dose after 24hrs. Even though, affection in the area postrema could not be the main cause of the renal and respiratory dysfunction in ophidic accidents, also it is important to know all the possible venom effects because many of the brain injuries are not reported or underestimate to affecting the patient health.

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## STATEMENT OF COMPETING INTERESTS

None declared.

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