Crotalus lepidus lepidus Venom Produces Hemolysis and Lipid Peroxidation in Human Erythrocytes *in vitro*

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Abstract

The hemolytic and lipid peroxidative effects of crude venom from *Crotalus lepidus lepidus*, a rattle snake found in the Northeast of Mexico and southeast of the United States, were investigated in human erythrocytes *in vitro*. Significant concentration-dependent effects were found on both hemolysis (evaluated as release of hemoglobin) and lipid peroxidation (as a common index of oxidative damage to membrane lipids) in the red blood cells. These results suggest that hemolysis was produced with the involvement of oxidative stress as a potential mechanism of toxicity in the erythrocytes.

Keywords: Hemolysis; Lipid Peroxidation; *Crotalus lepidus lepidus* Venom

Abbreviations: TBA: Thiobarbituric Acid; TCA: Trichloroacetic Acid; MDA: Malondyaldehide; (C.l. lepidus): *Crotalus Lepidus Lepidus*

Introduction

Snake venoms are among the best pharmacologically characterized natural toxins, chiefly because of their deleterious effects on humans [1]. Snake venoms are toxic secretions, which are complex mixtures of molecules of different biochemical nature, with a predominance of proteins, many of which are endowed with enzymatic activities [2]. The most common snake venom enzymes include acetylcholinesterases, L-amino acid oxidases, serine proteinases, metalloproteinases and phospholipases A_2 [2]. Some phospholipases A_2 cause a wide variety of pharmacological effects such as pre-synaptic or post-synaptic neurotoxicity, miotoxicity, anticoagulant and hemolytic activities [3,4]. PLA_2 catalyzes the specific hydrolysis of ester bonds at the C_2 position of 1,2-diacyl-3-sn-glycerophospholipids with release of free fatty acids. Thus, phosholipases A_2 are able to disrupt the phospholipid packings from several types of biological membranes, leading to cell lysis [5]. There is some evidence that animal venoms can induce oxidative stress. For example, El Asmar et al. and Dousset et al. [6,7] have reported that the increase in polyunsaturated fatty acids following envenomation by scorpion venom may lead to an increase in the rate of lipid peroxidation, which might be responsible for tissue damage. In the case of snakes, the venom of *Echis piramidum* causes lipid peroxidation in different organs of mice [8] and the venom of *Bothrops* induces renal tubular toxicity mediated in part by lipid peroxidation [9]. Currently, lipid peroxidation is one of the most important organic expressions of oxidative stress induced by the free radicals produced during various types of xenobiotic exposures or pathological conditions, animal venoms included. The potential consequences of the peroxidative process of membrane lipids include loss of polyunsaturated fatty acids, decreased lipid fluidity, altered membrane permeability, effects on membrane-associated enzymes, altered ion transport, release of material from subcellular compartments, and the generation of cytotoxic metabolites or lipid hydroperoxides [10]. Furthermore, vertebrate red blood cell membranes have a high content of unsaturated lipids as well as iron in hemoglobin, one of the most powerful catalysts capable of initiating lipid peroxidation [11]. In addition, erythrocytes are anucleated cells and therefore lack protein synthetic machinery, they cannot

replace many cellular components, so oxidative damage may induce a permanent alteration in the red cells [11]. In this study, we evaluated hemolytic response and lipid peroxidation of the crude venom from the rattlesnake *Crotalus lepidus lepidus* on isolated human erythrocytes.

Materials and Methods

Chemicals

Sodium Citrate, Anhydrous Dextrose, Sodium Chloride, Citric Acid, Thiobarbituric Acid (TBA), Trichloroacetic Acid (TCA) were all purchased from J.T. Baker (Center Valley, PA). All other reagents were from Sigma (St. Louis, MO).

Preparation of venom extract

The venom of *Crotalus lepidus lepidus* was purchased from the laboratory of National Natural Toxins Research Center at Texas A&M University-Kingsville. Venom solution was prepared using 10 mg of lyophilized venom in 5.0 mL of Alsever's solution pH 6.4 (dextrose 0.116 M, NaCl 0.071 M, sodium citrate 0.027 M, and citric acid 0.002 M), then, centrifuged at 12,000 g for 10 min at 4°C. The pellet was discarded and the supernatant was aliquoted and stored at -20°C until use. Protein concentrations were determined in samples by the method of Bradford (1976) [12] with bovine serum albumin as a standard.

In vitro hemolysis assay

The present study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Chemistry of the Autonomous University of Coahuila, Mexico. The hemolysis test was performed using human whole blood from healthy non-smoking donors with permission, following guidelines for studies using human samples. In briefly, blood was collected in heparinized-tubes, was centrifuged at 3000 rpm for 4 minutes at 4°C. The pellet was washed three times with cold Alsever's solution. The supernatant was then removed and 100 mL of the purified erythrocytes were diluted 1: 99 with Alsever's solution. Then, 150 mL of this suspension was suspended in Alsever´s buffer and taken for the curve-response experiments (total volume 1500 ml). This suspension of red blood cells was always freshly prepared and used within 24h after collection. Crude venom concentrations were tested: 0.250, 0.500, 1.0 and 2.0 mg/mL. The tubes were gently mixed in a rotator shaker, and then incubated at 36.5°C ± 1°C within a shaking water bath, for 60 minutes. Alsever's solution and deionized water were used as negative and positive controls, respectively. Each group contained three tubes. The samples were then centrifuged under 3000 rpm for 4 minutes to collect the supernatant. The absorbance (A) value of the hemoglobin released from the erythrocyte cells was measured spectrophotometrically at 415 nm (Thermo Spectronic Genesys 5). All trials were run three times. The absorbance obtained was correlated with a curve of human hemoglobin.

The experiments were run in triplicate and were repeated twice.

Determination of malondialdehyde (MDA)

Erythrocytes MDA concentration was determined by using the method described by Draper and Hadley (1990) based on TBA reactivity (TBARs) with some modifications. In this manner, 1.0 mL of supernatant of the erythrocytes suspension (it was prepared in the similar way as the hemolytic assay) was mixed with 45 mL of TCA (50%). The samples were centrifugated at 3000 rpm/10 min. Then, 115 mL of TBA (0.75 % in 0.1 M HCl) was added and put into a boiling water bath for 10 min. Tubes were centrifuged at 3,000 rpm for 10 min, and the absorbance was measured at 535 nm. Results were expressed as nmol TBARs/ mg protein. Concentration of MDA was calculated by a calibration curve using 1,1,3,3-tetraethoxypropane as a standard.

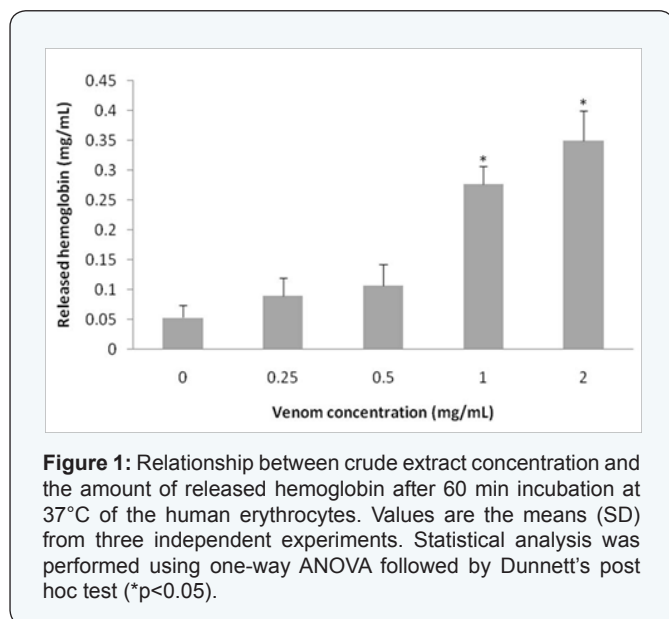### Phospholipase A_2 activity assay

The method of Araujo and Radvanyi [13] was used with some modifications. Venom solution (10 mg in 10 ml of PBS pH 7.4) was added to the reaction medium (1.5 mL) containing 15 mol phosphatydylcholine, 18 mol Triton X-100, 5mol calcium chloride, 80 mol phenol red and 7.5 mmol Tris at pH 7.9. Subsequently, the solution was incubated for 5 min at room temperature and added to a cuvette. Changes in the absorbance at 558 nm were followed during 5-8 min. Decrease in absorbance of phenol red by the acidification of medium was proportional to the liberation of fatty acids. The specific activity was calculated in Units (mM) of fatty acid per min. Controls included samples incubated with Alsever solution instead of venom. This assay provides information about presence and activity of phospholipases A_2 in the venom of *C. l. lepidus*.

Statistical Analysis

All data are expressed as mean SEM. All experiments were performed in triplicate and three independent experiments were carried out. For the statistical analysis of results, we employed one-way analysis of variance (ANOVA) followed by Dunnett's test for specific comparisons against control values. Values of $p < 0.05$ was accepted as significant levels.
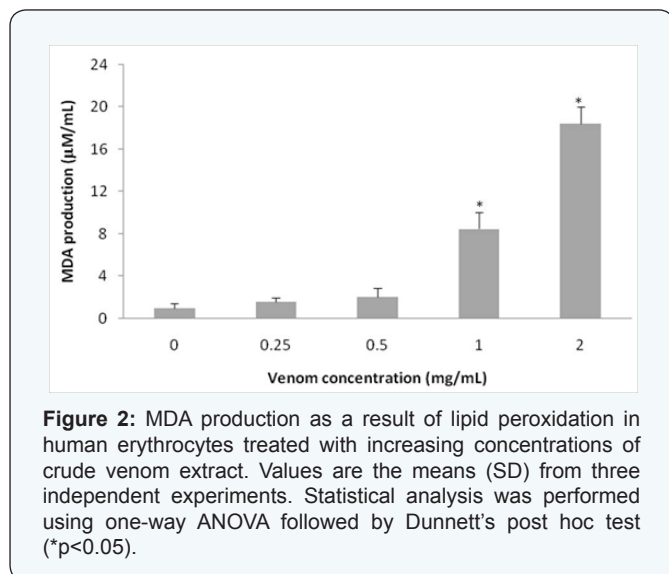
Results

The crude extract of *Crotalus lepidus lepidus* added to the red blood cells resulted in a concentration-dependent hemolytic response, expressed as percentage of released hemoglobin, obtaining the following values: 0 mg/mL (0.05 mg Hb/mL), 0.25 mg/mL (0.09 mg Hb/mL), 0.5 mg/mL (0.106 mg Hb/mL), 1 mg/mL (0.276 mg Hb/mL) and 2 mg/mL (0.35 mg Hb/mL) (Figure 1).

Figure 1: Relationship between crude extract concentration and the amount of released hemoglobin after 60 min incubation at 37°C of the human erythrocytes. Values are the means (SD) from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (*p<0.05).

Lipid peroxidation in erythrocytes

Significant increases in the levels of peroxidized products (measured as MDA production), with respect to control group, were found in the blood cells exposed to concentrations of the crude venom of *C. l. lepidus* of 1.0 mg/mL (8.5 mM/mL) and 2.0 mg/mL (20.12 mM/mL) (Figure 2).

Figure 2: MDA production as a result of lipid peroxidation in human erythrocytes treated with increasing concentrations of crude venom extract. Values are the means (SD) from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (*p<0.05).

Phospholipase A_2 activity

This assay was made to investigate presence and activity of phospholipases A_2 in the venom of *C. l. lepidus*. The phospholipase A_2 activity of the venom of *C.l. lepidus* (1mg/mL) was 0.041±0.0035 (mM/min). This concentration was chosen because it provoked the first increase in hemolysis and MDA production assays.

Discussion

The mottled rock rattlesnake (*Crotalus lepidus lepidus*) is one of four subspecies of *C. lepidus*. It is a relatively small rattle snake non aggressive although exhibits a wide range distribution includes southwest Texas, southeastern New Mexico and the Mexican Plateau to San Luis Potosi [14]. In venom of *C.l. lepidus* has been identified some partial sequences of metalloproteinases, galactose-specific lectins and phospholipases A_2, as well produces hemorrhagic and lethal activities in mice [15]. The clinical effects of a case envenomation by *C.l. lepidus* include hemorrhagic effects, ecchymosis, soft tissue swelling, pain, thrombocytopenia and other hematological alterations [16]. Our results indicate that crude venom of *C.l. lepidus* in concentrations of 1.0 and 2.0 mg/mL is effective at producing toxicity in human erythrocytes *in vitro*, as indicated by hemolysis and lipid peroxidation. We employed the hemolytic response as a suitable tool to evidence cytotoxicity since some authors have reported that hemolysis represents a very sensitive test to assess and characterize phospholipases of snake venom [17,18] and the TBARs assays as a common index of lipid peroxidation [19,20] to assess the involvement of free radical formation during the cytotoxicity elicited by the crude venom of *C.l. lepidus*. In addition, we determined that *C.l. lepidus* venom possesses PLA_2 activity, similar to the other *Crotalus lepidus* subspecies [15]. PLA_2 enzymes are unique calcium-dependent hydrolytic enzymes on phospholipids, liberating free fatty acid and lysophospholipid [5,21]. Phospholipid hydrolysis by PLA_2 enzyme also releases arachidonic acid whose metabolism results in the formation of potentially toxic reactive oxygen species and lipid peroxides. Red blood cells are particularly vulnerable to the attack by free radicals and therefore, they represent a suitable substrate to give evidence of oxidative stress [11,22]. Our results indicate that the venom of *C.l. lepidus* induces lipid peroxidation in human red blood cells. Erythrocytes lysis may be the end result of defects in the red blood cell membrane related to the peroxidative attack probably mediated by PLA_2, suggesting that the peroxidative actions might, at least partially, contribute to the hemolytic effect. Interestingly, Norris [16] reported a clinical case of a bite in a person of 30 years by a captive specimen of *C.l. lepidus* who provokes a reduction in hematocrit and hemoglobin values in the patient. Erythrocytes are also susceptible to oxidative stress due to unsaturated membrane phospholipids, and the presence of hemoglobin and other hematin compounds may also augment the process of lipid peroxidation [23]. In addition, the erythrocytes have a high content of iron in hemoglobin, a powerful catalyst capable of initiating lipid peroxidation [11]. It´s well known that lipid peroxidation produces oxidation of lipids, particularly polyunsaturated fatty acids and cholesterol [22,23]. In the lights of these results, the hemolytic and lipid peroxidation response to the crude venom was concentration-dependent, suggesting that part of the toxic action of the venom corresponds to the formation of free radicals mediated by PLA_2,

and hence the consequent changes in membrane structure after lipid peroxidation.

Conclusion

The findings of our study indicate that the crude venom from *C.l. lepidus* is able to induce hemolysis and lipid peroxidation in the isolated human red blood cells.

Acknowledgement

Conflicts of Interest: There is no conflict.

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