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VITAMIN E REDUCE HEMOLYSIS AND LIPID PEROXIDATION IN HUMAN ERYTHROCYTES CAUSED BY THE VENOM OF *CROTALUS LEPIDUS LEPIDUS*

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Abstract: The protective effect of vitamin E to reduce hemolysis and peroxidative damage in isolated human erythrocytes treated with venom of *Crotalus lepidus lepidus* was evaluated. Venom concentrations (250, 500, 1000 and 2000 mg/mL) and vitamin E (150, 600 and 2400 mg/mL) were incubated at 37°C for 1 hour. The results showed that vitamin E, in the concentration of 150 mg/mL, decreased the hemolysis induced with 1000 and 2000 mg/mL of venom, while 600 mg/mL of vitamin E was only effective with 2000 mg/mLof venom. In the case of peroxidation, only the concentration of 600 mg/mL of vitamin E reduced the production of malondialdehyde (as a peroxidation lipid index) with the concentrations of 1000 and 2000 mg/mL of the venom. On the other hand, the vitamin E concentration of 2400 mg/mL increased the hemolytic and peroxidative effects in the erythrocytes. Conclusion: the vitamin E has protective or pro-oxidative effects on human erythrocytes *in vitro*, in function of the concentration used.

Keywords: Vitamin E, hemolysis, lipid peroxidation, *Crotalus lepidus lepidus* venom, human erythrocytes.

INTRODUCTION

Snake bites are a global public health problem due to both the incidence and the severity of their effects. The consequences of envenoming include dysfunction in tissue damage, mobility as well in hemostasis (Harrison *et al*., 2009).

Snakebite envenoming typically occurs in poor rural populations in tropical and subtropical regions, with the largest distribution of venomous snakes found in Africa, the Middle East, Asia, and South and Central America. In these areas, snakebite has a considerable socioeconomic impact on rural populations in developing countries (Gold *et al*., 2002; Gutiérrez *et al*., 2017). On the other

hand, natural compounds have been widely studied with the aim of complementing antiophidic serum therapy (de Moura Oliveira *et al*., 2016; Al-Sheikh *et al*., 2017).

Another important feature of the pathogenesis of snake envenomation is the production of reactive oxygen species (ROS). Generally, ROS generation can potentiate tissue damage at inflammatory sites after snakebite. While previous studies have witnessed its prevalence, the role of oxidative stress in poisoned patients remains an unrecognized scenario (Sunitha *et al*., 2015; Dong *et al*., 2020).

Snakebite envenoming is the result of venom, a mixture of different toxins, being injected into the body. Such venoms contain a complex mixture of enzymes, non-enzymatic proteins, peptides, lipids, nucleotides, and various ions. Among the classes of enzymes responsible for the deleterious effects are metalloproteases, serine proteases and L-amino acid oxidases and phospholipases A2 (PLA2s), among others (Seifert *et al*., 2022).

In particular, phospholipase type A2 (PLA2) is widely present in elapid and viperid snake venoms (Gutiérrez and Ownby, 2003; Gutiérrez *et al*. 2009), including *Crotalus lepidus lepidus* venom (Martínez-Romero *et al*., 2013; Montañez-Rodríguez *et al*., 2016). PLA2 exhibit a high degree of toxic activities, at presynaptic or postsynaptic sites, skeletal and cardiac muscles, coagulation factors, platelet aggregation, as well as hemorrhagic, cytolytic and hemolytic activities (Larréché *et al*., 2021; Gutierrez *et al*., 2021). In addition, specific components of the venom of snakes such as metalloproteinases, three-fingered toxins (3FTx), L-amino acid oxidase and phospholipases A2 are known to induce oxidative stress in biological models *in vitro* and in *vivo* (Sunitha *et al*., 2015).

For example, the venom of *Echis piramidum* causes lipid peroxidation in different organs of mice (Al Asmari *et al*., 2006), and the venom of *Bothrops* induces renal tubular toxicity mediated in part by lipid peroxidation (de Castro *et al*., 2004). In other case, human blood incubated with Russell's viper venom can stimulate H_2O_2 and malondialdehyde production (Santhosh *et al*., 2013).

In cells, venom of the rattlesnake *Crotalus molossus nigrescens* induces hemolysis and oxidative stress on human erythrocytes (Meléndez-Martínez *et al*., 2017). In this manner, 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.

On the other hand, tocopherols administered either *in vitro or in vivo* inhibited the hemolysis and peroxidative damage (Liu, 2006; Ozkanlar and Akcay., 2012).

The present study evaluated the inhibitory potential of vitamin E on the hemolytic response and lipid peroxidation of the crude venom from the rattlesnake *Crotalus lepidus lepidus* on isolated human erythrocytes.

MATERIALS AND METHODS

REAGENTS

Sodium citrate, anhydrous dextrose, sodium chloride, citric acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA) were all purchased from J.T. Baker (Center Valley, PA). Vitamin E and the other reagents were obtained from Sigma (St. Louis, MO).

VENOM EXTRACT

Crotalus lepidus lepidus venom was purchased from the laboratory of the National Research Center for Natural Toxins at Texas A&M University-Kingsville. The venom solution was prepared using 20 mg of lyophilized venom in 10 mL of Alsever pH 6.4

solution (0.116 M dextrose, 0.071 M NaCl, 0.027 M sodium citrate, and 0.002 M citric acid), then centrifuged at 12,000 rpm for 10 min at 4°C. The pellet was discarded, and the supernatant was aliquoted and stored at -80°C until use. Protein concentrations were determined in the samples by the Bradford method with bovine serum albumin as standard.

HEMOLYTIC ASSAY WITH VENOM EXTRACT AND VITAMIN E

This study was approved by the Animal Experimentation Ethics Committee 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 the guidelines for studies with human samples. Briefly, blood was collected in heparinized tubes, centrifuged at 2500 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 µL of the purified erythrocytes were diluted 1:99 with Alsever's solution. Then, 150 µL of this erythrocyte suspension was added in Alsever's buffer and taken for response curve experiments in a 24-well culture plates. (total volume 1500 µL). This red blood cell suspension was always prepared fresh and used within 24 hours of collection.

Venom concentrations (250, 500, 1000 and 2000 mg/ml) and vitamin E (150, 600 and 2400 mg/mL) were incubated with human erythrocytes at 37°C for 1 hour. Alsever's solution and deionized water were used as negative (0% hemolysis) and positive (100% hemolysis) controls, respectively. After incubation, samples were centrifuged at 2500 rpm for 4 min to collect the supernatant. The absorbance value (A) of hemoglobin released from the erythrocytes was measured spectrophotometrically at 415 nm (Thermo Spectronic Genesys 5). All tests were performed two times.

The hemolysis rate (HR) was calculated as follows: HR (%) = (A of experimental group - A of negative control group)/(A of positive control group - A of negative control group) X 100 %. The experiments were performed in triplicate and repeated twice.

QUANTIFICATION OF MDA BY TBARS ASSAY AS AN INDEX OF LIPID PEROXIDATION

Thiobarbituric acid reactive substances (TBARS) are a common way to measure lipid peroxidation products in cells. This assay measures malondialdehyde (MDA), which is a split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of lipid substrates. The MDA reacts with thiobarbituric acid (TBA) forming a pink chromogen (TBARS), which is measured at 532-535 nm (Kumar *et al*., 2018).

Venom doses (250, 500, 1000 and 2000 mg/mL) and vitamin E (150, 600 and 2400 mg/mL) were incubated at 37.0°C for 1 hour. Then, 1 mL of the supernatant of the erythrocyte suspension (prepared similarly to the hemolytic assay) was mixed with 45 mL of TCA (50 %). The samples were centrifuged at 3000 rpm/10 min. Then 115 mL of TBA (0.75 % in 0.1 M HCl) was added and placed in a boiling water bath for 10 min. The tubes were centrifuged at 3000 rpm for 10 min and the absorbance was measured at 535 nm (Thermo Spectronic Genesys 5). The results were expressed as nmol TBAR/mg protein. A positive control with hydrogen peroxide was used. The concentration of MDA was calculated using tetraethoxypropane as an external standard.

STATISTICAL ANALYSIS

For statistical analysis of the results, we used a one-way analysis of variance (ANOVA) followed by Dunnett's test for specific comparisons with control values. Values of *p<0.05 were accepted as significant level.

RESULTS

HEMOLYTIC ACTIVITY IN HUMAN ERYTHROCYTES

The crude extract of *Crotalus lepidus lepidus* caused hemolysis in the erythrocytes at concentrations of 1000 (21.47 %) and 2000 mg/mL (37.21 %) (Table 1).

The addition of 150 mg/mL of vitamin E to the erythrocytes treated with the venom reduced the hemolytic effect at venom concentrations of 1000 and 2000 mg of venom (12.16 % and 20.32 %, respectively). While, the addition of vitamin E, at–concentration of 600 mg/ml, only had an antihemolytic effect in venom concentration of 2000 mg/mL (15.60 %). However, the addition of 2400 mg/mL of vitamin E to the erythrocytes treated with venom, caused and pro-hemolytyc effect in the venom concentrations of 1000 and 2000 mg/mL (33.50% and 42.59 %, respectively).

LIPID PEROXIDATION IN ERYTHROCYTES

Significant increases in the levels of peroxidized products (measured as nmol MDA/mg protein), with respect to control group, were found in the blood cells exposed to concentrations of the crude venom of *Crotalus lepidus lepidus* of 1000 mg/mL (17.88 nmol MDA/mg protein) and 2000 mg/mL (16.12 nmol MDA/mg protein), respectively (Table 2).

The addition of 150 mg/ml of vitamin E to the erythrocytes treated with the venom reduced the oxidative effect at venom concentrations of 1000 and 2000 mg/mL of venom (7.34 and 8.23 nmol MDA/mg protein, respectively), while the addition of vitamin E, at dose of 600 mg/mL, only had an antihemolytic effect in venom concentration of 2000 mg/mL (6.65 nmol MDA/mg protein). However, the addition of 2400 mg/mL of vitamin E to the erythrocytes treated with venom, caused and pro-hemolytic effect in the venom concentrations of 1000 and 2000 mg/mL (25.22 and 29.43 nmol MDA/mg protein, respectively).

DISCUSSIONS AND CONCLUSION

Our results indicate that crude venom of *Crotalus lepidus lepidus* at concentrations of 1000 and 2000 mg/mL is effective in producing toxicity in human erythrocytes *in vitro*, as indicated by hemolysis and lipid peroxidation. In addition, *C.l. lepidus* venom possesses PLA2 activity (Montañez-Rodriguez *et al*., 2016), and has been identified some partial sequences of phospholipases A_2 in the venom of *C.l .lepidus* (Martínez-Romero *et al*., 2013). The clinical effects of a case envenomation by *C.l. lepidus* include hematological alterations also (Norris, 2005).

Some of the lethal effects of snake venom are largely attributed to its active ingredient phospholipase A2 (PLA2). Phospholipid hydrolysis by the enzyme PLA2 releases arachidonic acid whose metabolism results in the formation of reactive oxygen species and potentially toxic lipid peroxides (Adibhatla *et al*., 2003; Neuzil *et al*., 1998). Our results indicate that venom induces hemolysis and lipid peroxidation in human red blood cells. Erythrocyte lysis may be the result of red cell membrane defects related to peroxidative attack probably mediated by PLA2, suggesting that peroxidative actions could at least partially contribute to the hemolytic effect (Nethery *et al*., 2000).

	Hemolysis %				
Sample (mg/mL)	Venom concentration (mg/ml)				
	0	250	500	1000	2000
Negative control	0.52 ± 0.78				
Vitamin E (150)	0.33 ± 0.18				
Vitamin E (600)	0.39 ± 0.89				
Vitamin E (2400)	0.52 ± 0.76				
Venom	0.62 ± 0.32	0.70 ± 1.35	1.02 ± 0.45	21.47 ± 2.45*	37.21 ± 1.07*
Venom + Vitamin E (150)	0.92 ± 0.65	0.22 ± 0.93	0.24 ± 1.26	12.16 ± 0.87*	20.32 ± 2.22*
Venom + Vitamin E (600)	0.91 ± 0.96	0.04 ± 1.42	0.06 ± 1.73	23.18 ± 1.55*	15.60 ± 1.92*
Venom + Vitamin E (2400)	0.66 ± 0.99	0.05 ±1.26	1.42 ± 0.95	33.50 ± 2.18*	42.59 ± 2.09*
Positive control	100 ± 2.26				

Table 1. Relationship between vitamin E and crude extract concentration and the hemolysis percentage after 60 min incubation at 37 °C of the human erythrocytes.

Values are the means (SD) from two independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (*p<0.05).

	Lipid peroxidation				
Sample (mg/ml)	nmol MDA/mg protein				
	0	250	500	1000	2000
Negative control	0.67± 0.34				
Vitamin E (150)	1.28 ± 0.73				
Vitamin E (600)	0.76 ± 0.98				
Vitamin E (2400)	1.52 ± 0.88				
Venom	1.01 ± 0.22	1.02 ± 0.85	1.32 ± 0.48	17.88 ± 2.23*	16.12 ± 0.43*
Venom + Vitamin E (150)	0.23 ± 0.87	0.82 ± 0.78	0.98 ± 0.82	7.34 ± 1.98*	8.23 ± 2.62*
Venom + Vitamin E (600)	1.22 ± 0.45	1.11 ± 0.89	0.06 ± 1.73	15.67 ± 2.11*	6.65 ± 1.57*
Venom + Vitamin E (2400)	0.98 ± 0.78	1.46 ± 0.65	1.8 ± 1.09	25.22 ± 1.98*	29.43 ± 2.06*

Table 2. Relationship between vitamin E and crude extract concentration and the lipid peroxidation concentration after 60 min incubation at 37 °C of the human erythrocytes.

Values are the means (SD) from two independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (*p<0.05).

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 (Chiu *et al.*, 1982). 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 (Chiu *et al.*, 1982).

Viper venom mix contains high amounts of snake venom metalloproteinases that damage extracellular matrix proteins, especially at the base of the membrane that provides mechanical stability to microvessels. Rupture of the microvessel walls induces hemorrhage, which may contribute to red blood cell extravasation and hemolysis associated with the release of hemoglobin and redox-active free iron that triggers oxidative stress in target organs (Gutierrez *et al.*, 2016).

In this manner, growing information suggests that oxidative stress is a key

player involved in the pathophysiology of snakebite envenomation. Following venom administration to the body, the enzymatic and non-enzymatic components of the venom mixture induce deleterious actions that disrupt redox homeostasis and hematologic abnormalities. Also, venom-induced oxidative stress is involved in local and systemic complications in the initial stages of envenoming (Warrell, 2010; Sunitha *et al*., 2015; Resiere *et al*., 2022).

In our results, with regard TBARS assay, it was possible to observe an increase in MDA as the venom concentration increased. Meléndez et al., 2017, also found that venom of *Crotalus molossus nigrescens* is an inducer of lipid peroxidation in erythrocytes.

On the other hand, vitamin E is transported in the blood by lipoproteins and acts as an effective inhibitor of lipid peroxidation in cell membranes (Liu 2006; Ozkanlar and Akcay, 2012). For example, the protective mechanisms against damage induced by *Bothrops* venoms are probably the result of the antioxidant properties of these vitamins, as well as hydrophobic interactions between vitamin E molecules and venom toxins (de Moura Oliveira *et al*., 2016).

Also, Muherjee *et al*. (1998) demonstrated that vitamin E is capable of inhibiting lipid peroxidation induced by snake venoms in human erythrocyte cell membranes, and this is one of the probable mechanisms responsible for reducing the activity induced by PLA2s of the venoms.

In light of our results, the hemolytic and lipid peroxidation response to crude venom was concentration-dependent, suggesting that part of the toxic action of the venom could be related to the presence of phospholipases A2 in the venom (Martínez-Romero *et al*., 2013; Montañez *et al*., 2016). Addition of vitamin E could have a protective effect reducing harmful activity of the PLA2s of the venom of *Crotalus lepidus lepidus* on the erythrocytes, or pro-oxidative effects on the red blood cells, depending on their concentration.

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