

Isolation and Characterization of a Myotoxic Fraction from *Cerastes Vipera* Snake Venom

Keywords: Phospholipase A₂ - Myotoxin; Viper venom; *Cerastes vipera*; Myonecrotic action

Abstract

The aim of this study was to evaluate the biological effects of crude *Cerastes vipera* venom and some phospholipase A₂ (PLA₂) activity containing fraction (CV-PL-F4). Furthermore, the current study examined the antimyotoxic action of dexamethasone, vitamins (Vit.) E and C and trypsin against the isolated fraction having the highest PLA₂ activity and crude venom. In the present study, a PLA₂, designed CV-PL-F4, was isolated from *Cerastes vipera* snake venom through Sephadex G-75, and a screening for PLA₂ activity (via an indirect hemolysis assay) guided the search for a myotoxin. In conclusion, dexamethasone and Vit E are potentially useful in treating the myotoxic effect induced by *Cerastes vipera* crude venom and their CV-PL-F4 fraction.

Introduction

The bite of the viper *Cerastes vipera* has a serious medical problem. *C. vipera* venom contains a complex mixture of enzymes [1] including phospholipase A₂ [2] and proteases [3,4]. Some clinical effects following snake bite are due to PLA₂ and proteases which are among the major components of viper venoms. Although these enzymes share a common cytotoxic activity and similar structural features with non-toxic mammalian pancreatic enzymes, they also promote a wide variety of pathological symptoms in animals, such as cardiotoxicity, myotoxicity, anticoagulant activity and hemolysis [5-7]. Since phospholipase A₂ is almost invariably the most toxic component of the venoms, it probably plays an important role in mortality and morbidity following snakebite [5,8]. The primary target of PLA₂ is the cell membrane inducing the hydrolysis of its phospholipids [9] as the hydrolysis of erythrocyte membrane [10-12] and the damage of the muscle cell membrane inducing myonecrosis [13-15]. A few PLA₂ of the vipers have been isolated and purified [2,9,16,17]. The study of myotoxicity of *Cerastes vipera* venom has received relatively little attention [18,19]. The purposes of the present study are (a) to isolate a fraction having a myonecrotic effect with PLA₂ activity from *Cerastes Vipera* venom, (b) to evaluate the biological properties of the fraction, and (c) to investigate the antimyotoxic action of dexamethasone, Vit E and C and trypsin for the isolated fraction.

Materials and Methods

Venom and chemicals

Cerastes vipera venom was extracted from adult vipers collected from Aswan area and maintained in the Venom Research Lab, Department of Zoology, Faculty of Science, Ain Shams University. Venom was collected from the vipers every four weeks for a six-month period by allowing the vipers to bite through a rubber glove



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covering a glass funnel that emptied into a glass tube in the ice. The venom was lyophilized and stored at 4°C until used. Sephadex G-75, phosphatidylcholine, casein (fat-free), sodiumdodecylsulfate (SDS), 2-mercaptoethanol, acrylamide, coomassie brilliant blue R250, trypsin, bovine serum albumin dexamethasone, Vit E, vitamin C and protein molecular weight markers were obtained from Sigma Chemical Co.

Animals

Male CD-1 White mice weighing approximately 20-25 g were housed in specially designed cages and maintained in conditions of good ventilation, normal temperatures, and humidity ranges. Mice were housed 5 animals in each cage to avoid crowding under conditions of equal periods of light and dark in the animal house (12 hr light and 12 hr dark cycle). Mice were fed on standard pellets, containing all nutritive elements (proteins, fats, carbohydrates, vitamins, salts and minerals). Drinking water and food were provided ad libitum throughout the period of study. All experiments followed the ethical standards for animal experiments in toxicological research recommended by the International Society of Toxinology [20].

Fractionation of *Cerastes vipera* venom

Samples of lyophilized *Cerastes vipera* venom (110 mg) were reconstituted in 5 ml 0.03 M ammonium acetate buffer, pH 4.6 and applied to a 2.5x75 cm column (Pharmacia Biotech, Piscataway, NJ, USA) packed with Sephadex G-75 equilibrated with the same buffer and eluted at a flow rate of 1 ml/min using an EP-1 Econo Pump (Bio-Rad Laboratory, Hercules, CA, USA). Five-milliliter fractions were collected, and elution profile was monitored at 280 nm.

Hemolytic activity of *Cerastes vipera* venom and its fraction

Hemolytic activity of the crude venom and its isolated venom fraction was determined by measuring the release of hemoglobin from rabbit erythrocytes enhanced by the addition of phosphatidylcholine. This indirect hemolytic protocol was performed in glass tubes. 10,20,40 and 60 µg of the crude venom and its isolated fraction were added to a prewarm (to 37 °C) assay mixture containing 0.1 ml of phosphatidylcholine suspension (10 mg /L), 1 ml of rabbit erythrocytes (2.5% v/v) and 2.9 ml of Tris buffer (1 mM, pH 7.5)

containing NaCl (100 mM), KCl (100 mM) and CaCl₂ (10 mM). After incubation for 1 hr, the tubes were centrifuged at 2500 g for 15 min using IEC-Centra-4R cooling centrifuge (I. E. Company, USA). The supernatant was used to estimate the degree of hemolysis at 540 nm. Hemolytic unit (HU₅₀) value is the amount venom or a fraction caused 50% hemolysis and was calculated according to the method of [21].

Myotoxic activity of the venom and fractions

Ten fractions obtained from the viper venom were tested for their ability to induce myonecrosis by intramuscular (i.m.) injection into male white CD-1 mice (n=3). After 3 hours, the mice were anesthetized with ether and killed by cervical dislocation. Samples of serum and muscle tissue were taken and processed as described by Rodrigues et al. [22]. Myotoxicity was assessed by the increase in serum Creatine Kinase (CK) activity, in addition to the determination of myonecrotic index using histological analysis.

Essay of PLA₂ of *Cerastes vipera* myotoxin

The PLA₂ activity of the CV-PL-F₄ myotoxin fraction was determined using the method described by Joubert and Taljaard [23]. 1 ml of egg yolk suspension was mixed with 4.0 ml 0.1 M Tris buffer, pH 8, and 0.1 ml of the enzyme and incubated at 30 °C. The decrease of the turbidity of the suspension was recorded at 740 nm. The absorbance change between 5 min and 15 min intervals was taken as the relative amount of enzyme activity. One unit was defined as the amount of enzyme which produced a decrease of 0.01 in absorbance in 10 min.

Proteolytic activity assay of *Cerastes vipera* myotoxin

Proteolytic activity of CV-PL-F₄ was estimated with casein (fat-free, BDH, England) using the method described by Mandelbaum et al. [24]. The reaction mixture contained 1 ml of a 2% casein solution in 0.2 M Tris-HCL buffer, 8 mM CaCl₂, at pH 8.8 and 1 ml of *Cerastes vipera* myotoxin solution. After 30 min incubation at 37 °C, 3 ml of 5% trichloroacetic acid was added to the mixture and the precipitate was removed by filtration. The amount of acid soluble products was measured in 1 ml of the filtrate by the method of Lowry et al. [25]. The specific caseinolytic action was defined as absorbance/min/mg of protein at 750 nm.

Electrophoresis

Homogeneity and molecular weight of the crude venom and CV-PL-F₄ fraction were determined by slab SDS-PAGE using the apparatus of Bio/Phoresis™ (Bio Gene Limited, England). The protein samples were dissolved in a sample buffer containing 0.01 M Tris-HCl (pH 8.6) and 10% SDS with 5% 2-mercaptoethanol. After heating at 100 °C for 5 min, the samples were subjected to electrophoresis following the procedure described in the Bio Gene Technical Bulletin No. 110. Slab gels were stained using Coomassie brilliant blue R 250. M.W. calibration markers were subjected to SDS-PAGE electrophoresis together with the viper venom and CV-PL-F₄. The reference proteins used were: β-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (45,000), phosphorylase b (97,000) and myosin H-chain (200,000).

Protein determination

Protein concentrations of crude venom, column eluates, and isolated fractions were measured by the method of Lowry et al. with bovine serum albumin as a standard or by spectrophotometric absorption at 280 nm [25].

LD₅₀ determination

The amount of a venom or its fractions that are sufficient to kill 50 percent of a population of animals usually within a certain time is called median lethal dose (LD₅₀). LD₅₀ was determined by Intraperitoneal (i.p.) injection of different CV-PL-F₄ doses into male CD-1 White mice. The survival time for each mouse was recorded up to 24 hr. LD₅₀ was calculated according to the method described by Meier and Theakston [26].

Experimental protocol

a- Myotoxic effect of *Cerastes vipera* venom and CV-PL-F₄: *Cerastes vipera* venom (10 µg/ 25 g mice) or myotoxin CV-PL-F₄ (30 µg/ 25 g mice) was dissolved in 0.1 ml of 0.85% NaCl. The dose of *Cerastes vipera* venom used in this study was equal to the sub LD₅₀, where the LD₅₀ of the venom was equal to 15.62 µg/ 25 g mouse. CD-1 mice (64 animals) were divided into eight groups, each of eight animals. Six animal groups were i.m. injected in the dorsolateral aspect of the gastrocnemius muscle with either venom or CV-PL-F₄ to study the effect of the viper venom and CV-PL-F₄ for three-time intervals. Mice injected with the crude venom or CV-PL-F₄ was sacrificed after 1, 3 and 24 hr. The other two animal groups were injected with 0.85% NaCl only and were used as a control.

b- Antimyotoxic effect of dexamethasone, vitamins E, and C and trypsin: Ten animal groups (80 mice) were used each of eight animals. Two animal groups injected i.m. with the saline solution containing 10 µg of *Cerastes vipera* venom or 30 µg CV-PL-F₄ and were used as control groups. Dexamethasone (50 µg), Vit E (0.1 mg) and C (4 mg) were mixed with 10 µg *Cerastes vipera* venom or 30 µg CV-PL-F₄ and i.m. injected in the dorsolateral aspect of the gastrocnemius muscle of six groups of mice respectively. Crude venom (10 µg) or CV-PL-F₄ (30 µg) was incubated for 1 hr at 37 °C with 50 mg trypsin to hydrolyze the proteins of the crude venom or CV-PL-F₄. The mixtures were centrifuged for 15 min at 3000 rpm. The supernatants of the venom and myotoxin were i.m. injected in the last two animal groups respectively. The ten animal groups were sacrificed after 3 hr.

Measurements of serum CK activity, serum creatinine level, and myonecrotic index: Blood was collected from all animal groups of experiments (a) and (b) to separate serum. Serum CK activity and creatinine level were determined using the method described by Szasz et al. and Annino and Giese respectively [27,28]. Evaluation of myotoxicity of *Cerastes vipera* venom and CV-PL-F₄ was carried out by measuring serum CK activity using a commercial colorimetric kit (Randox Laboratories Ltd. UK). The myonecrotic index was determined also from thick sections of muscle tissue stained with hematoxylin-eosin and examined with the light microscope according to the method described by Rodrigues et al. [22]. Damage and vacuolated muscle fibers were counted for each of three sections of tissue per mouse to give a final vacuolation index for each mouse. Usually, 2000 cells were counted per mouse. A necrosis Index was calculated as a number of necrotic cells/total number of cells.

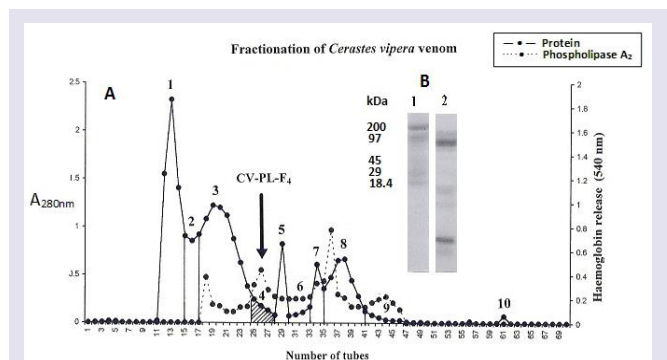


Figure 1: (A) Separation of *Cerastes vipera* venom by gel filtration on Sephadex G- 75 (2.5 x 75 cm column). Samples (110 mg) of crude venom, dissolved in 5 ml of 0.03 m ammonium acetate buffer (pH 4.6), were applied to the column. The column was eluted with ammonium acetate buffer and tubes of 5 ml were collected. The protein content of each tube was measured at 280 nm (—■—) and the degree of hemoglobin release was assayed at 540 nm (.....●.....). Vertical lines indicate limits of the pools 1 to 10. The fraction CV-PL-F₄ (shaded peak) had myotoxic activity and was used in the present study. (B) SDS polyacrylamide gel electrophoresis of *Cerastes vipera* venom. Lane 1 contain β-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (45,000), phosphorylase b (97,000) and myosin H-chain (200,000) as molecular weight markers. Lane 2 contains *Cerastes vipera* venom.

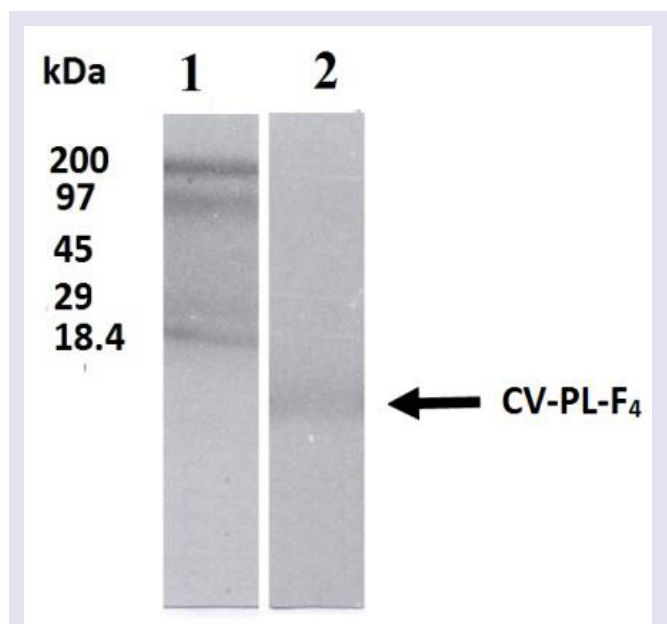


Figure 2: SDS polyacrylamide gel electrophoresis of CV-PL-F₄ myotoxin of *Cerastes vipera* venom. Polyacrylamide gels (12%) were run using Bio/Phoresis™ unit (Bio Gene Limited, England). Protein bands were detected by Coomassie brilliant blue R-250. Lane1 contain β-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (45,000), phosphorylase b (97,000) and myosin H-chain (200,000) as molecular weight markers. Lane 2: contains CV-PL-F₄ myotoxin.

Statistical analysis: The significance of the differences between the means of two experimental groups was determined by the Student's t-test. In some cases, the correlation coefficient between two sets of data was analyzed using one-way analysis of variance [29]. Values of P < 0.05 were considered statistically significant.

Results

Fractionation of *Cerastes vipera* venom

The fractionation of the crude *Cerastes vipera* venom showed ten fractions on Sephadex G-75 (Figure 1). The percentage of the yield of these fractions is shown in (Table 1). The first fraction was the greatest yield isolated toxin (34.5%) of the venom while the higher protein content was recorded in the third isolated fraction (818.3 mg/g). The hemolytic activity of the eluents of the venom after gel filtration appeared in fractions from the third to the tenth fraction. Fraction 4 (CV-PL-F₄) had the highest PLA₂ (Figure 1), the highest percentage increase in the rate of serum CK release (Table 1) and the highest proteolytic activities (Table 2).

Characterization of a phospholipase A₂ fraction (CV-PL-F₄) isolated from *Cerastes vipera* venom

Analysis of CV-PL-F₄ fraction with SDS-PAGE under reducing conditions showed the presence of a single band (Figure 2). The M.W. of this protein fraction was estimated to be 14,600. When mice were i.m. injected with 30 μg / 25 g of the CV-PL-F₄, they did not show any neurotoxic symptoms such as convulsions, respiratory distress or

Table 1: Protein content of *Cerastes vipera* venom and isolated fractions and the release of CK.

	Protein *	Yield (%)	% increase in the rate of serum CK release®
Crude venom	927.81		255
Fractions			
1	726.8	34.5	97
2	379.6	2.2	55
3	818.3	18.3	88
4	214.8	2.5	175
5	128.8	6.6	92
6	53.6	2.7	91
7	84.2	6.7	108
8	52.4	13.5	94
9	13.8	3.5	54
10	55.4	2.3	48

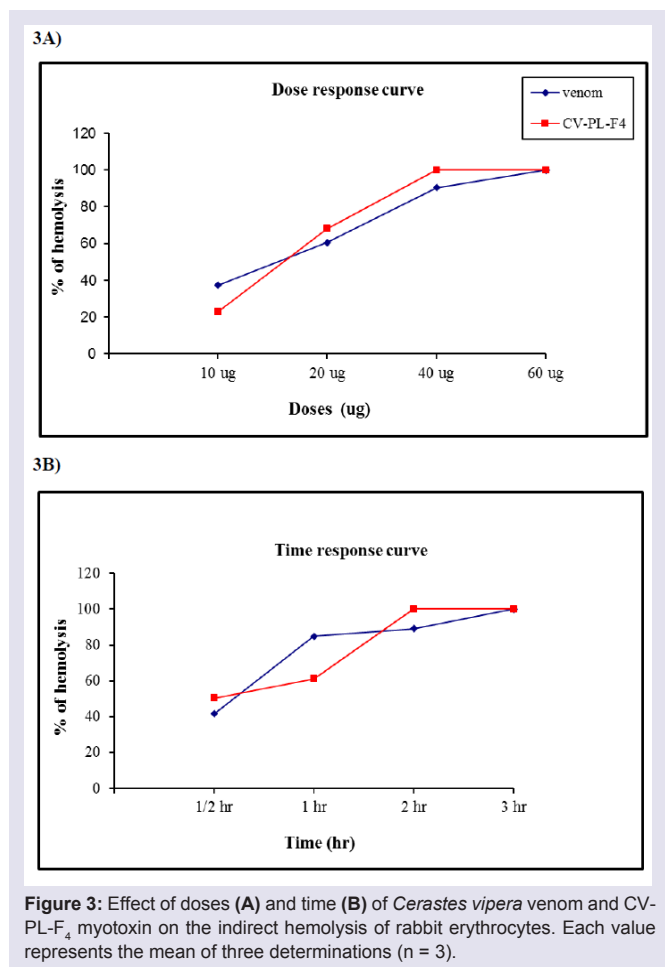
* Protein content is expressed as mg protein /g venom or fraction.

® Percent of serum CK release after 3 hr of i.m. injection of 15 μg venom / 25 g mouse or 50 μg CV-PL-F₄ myotoxin in comparison with the control serum CK activity; 411.9 ± 48.6 U/L (Mean ± SD).

Table 2: Biochemical and biological properties of the CV-PL-F₄ fraction isolated from *Cerastes vipera* venom.

CV-PL-F ₄	Property
14600	- Molecular weight
-	- Lethality
48	- Survival time (hr)
1.78 ± 0.21	- Proteolytic activity (D A ₇₅₀ / 3 min / mg)
23.15 ¶	- Protein yield (%)
0.014	- Phospholipase A ₂ activity (D A ₉₂₅ / 10 min)
+ (in muscle and lung)	- Hemorrhagic activity
-	- Neurotoxicity

¶ % of the protein content of CV - PL - F₄ in comparison with that of the crude *Cerastes vipera* venom.



death. When the injection of CV-PL-F₄ was increased up to 150 µg/25 g, none of the mice showed any neurotoxic symptoms, nor did any mice die within 24,48 or 72 hr of the injection. Thus, the toxin does not appear to be highly lethal. CV-PL-F₄ fraction showed caseinolytic activity (Table 2). The protein content of this fraction was 214.8 mg/g (23.15% of the crude venom protein). At the site of CV-PL-F₄ injection, a hemorrhagic spot was observed on the inner surface of the skin and the outer surface of the gastrocnemius muscle “data not shown”. The electrophoretic pattern of *Cerastes vipera* venom showed nine bands with mol. wt. ranging from 1,406 to 180,360.

Hemolytic activity of the crude venom and CV-PL-F₄

The increasing effects of doses or time for the crude venom or CV-PL-F₄ were examined. Hemolysis induced by the viper venom (r = 0.962) and the myotoxin (r = 0.879) fraction was dose dependent (Figure 3). The doses of the venom and CV-PL-F₄ causing 50% hemolysis (UH50) were 14.69 and 16.77 µg. The Hemolytic activity of the venom (r = 0.831) and CV-PL-F₄ (r = 0.929) were also time dependent.

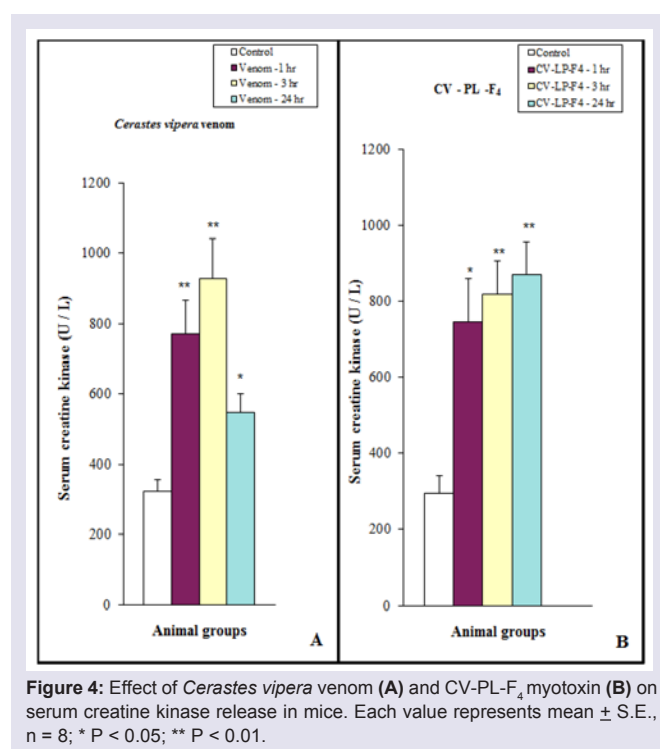
Myotoxicity of *Cerastes vipera* venom and CV-PL-F₄

The release of serum CK was significantly (P<0.01) increased after 1 and 3 hr of *Cerastes vipera* venom injection, but the enzyme activity after 24 hr was less than (P<0.05) the released after 1 and 3 hr. Figure

4A Shows the myonecrosis induced by i.m. injection of 10 µg venom/ 25 g mice using the myonecrotic index which includes all damaged cells (Figure 5). The presence of the necrotic muscle fibers and vacuolated fibers were used to estimate the amount of myonecrosis of *Cerastes vipera* venom and CV-PL-F₄ (Figure 5). The amount of myonecrosis of *Cerastes vipera* venom was observed (Figure 6) after 1 hr (50%), 3 hr (56%) and 24 hr (59%). Figure 4B shows that serum CK released following i.m. injection of 30 µg CV-PL-F₄ was significantly increased by 153% (P<0.05) after 1 hr, 178% (P <0.01) after 3 hr and 195% (P <0.01) after 24 hr. The myonecrotic index induced by CV-PL-F₄ was increased by 56%, 61% and 61% after 1, 3 and 24 hr of the fraction injection respectively (Figure 6).

Antimyotoxic effects of vitamins E and C, dexamethasone and trypsin

Dexamethasone or Vit (E or C) or trypsin was mixed with *Cerastes vipera* venom injection into mice, serum CK activity was significantly decreased (P < 0.05-0.01) compared with the venom-treated group but it was similar to those observed in control mice, indicating a complete inhibition of the CK release following venom injection (Figure 7A). Myonecrotic index of *Cerastes vipera* venom in mice treated with Vit E was decreased by 49% in comparison with that of mice treated only with the venom (Figure 8). This antimyonecrotic effect was higher on using Vit C (26% necrosis) in comparison with the necrotic effect of the venom. Also, dexamethasone and trypsin decreased the myonecrotic index of the venom to 34% and 36% respectively (Figure 8). Injection of a mixture of CV-PL-F₄ and Vit E showed complete inhibition of serum CK release. This effect was not similar when Vit C was injected instead of Vit E. Vit C partially (45%) antagonized the release of CK following injection of CV-PL-F₄. Dexamethasone and trypsin could abolish the ability of CV-PL-F₄ to



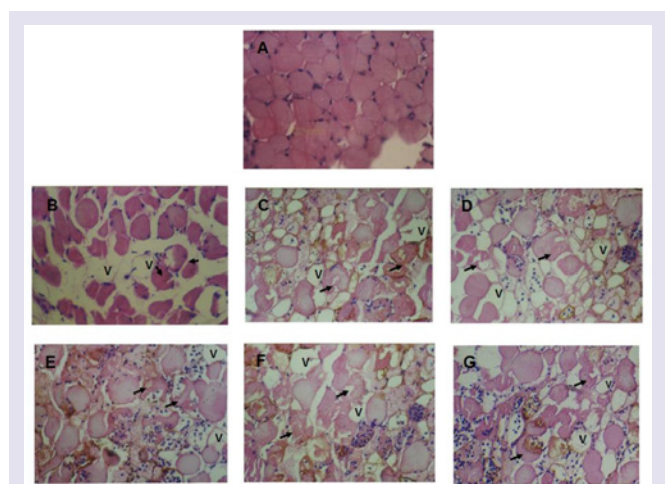


Figure 5: Photomicrographs of cross-sections of gastrocnemius muscle fibers taken after injection of *Cerastes vipera* venom or CV-PL-F₄ myotoxin. (A) Control muscle fibers from mice i.m. injected with 0.1 ml of 0.85% NaCl (PSS) as a control. Note the normal appearance of all muscle cells. (B), (C) and (D) Muscle fibers were taken from mice injected with 15 µg/25 g *Cerastes vipera* venom for 1, 3 and 24 hr respectively. (E), (F) and (G) Muscle fibers taken from mice injected with 30 µg/ 25 g CV-PL-F₄ myotoxin for 1, 3 and 24 hr respectively. Note the presence of the necrotic muscle fibers (N) and vacuolated fibers (V), 200 X.

release of CK from muscles (Figure 7B). Dexamethasone, Vit E and C, and trypsin abolished the myonecrosis of CV-PL-F₄ by decreasing the myonecrotic index to 34,40,31, and 24% respectively in comparison to the levels induced by CV-PL-F₄ only (Figure 8).

Effect of *Cerastes vipera* venom and CV-PL-F₄ on serum creatinine level

As shown in (Figure 9A), injection of *Cerastes vipera* venom (10 µg /25 g mice) or CV-PL-F₄ (30 µg / 25 g mice) induced a significant increase in serum creatinine during the experimental periods. While animals treated with the venom for 1 hr did not show any significant effect. On using Vit E, serum creatinine level of mice treated with the viper venom was comparable to that of the control mice (Figure 9A). Dexamethasone, Vit C, and trypsin failed to counterbalance the increase in serum creatinine of mice treated with the viper venom. In animals treated with CV-PL-F₄, only trypsin was able to decrease the high level of serum creatinine induced by the venom fraction (Figure 9B). Dexamethasone, Vit E, and C did not decrease the inductive effect of CV-PL-F₄ to increase the serum creatinine levels.

Discussion

The present results showed that ten fractions were isolated from *Cerastes vipera* venom. Eight fractions (from the third to the tenth fraction) with PLA₂ activity and two fractions (the first and the second fraction) without PLA₂ activity. The first and third peaks have the highest yield isolated toxin and protein content, respectively. The fourth peak (CV-PL-F₄) has the highest PLA₂ and proteolytic activities causing the most highly myotoxic fraction. This finding is similar to the observations of Labib et al. who isolated *Cerastes vipera* venom into 8 fractions and recorded that the first peak has the highest protease activity and lethality causing marked hemorrhages in mice [1]. PLA₂ snake venom usually showed indirect hemolytic activity

because promotes hydrolysis of lecithins to lysolecithins able to lyse RBC membranes [30]. The isolated PLA₂ from *Cerastes vipera* venom showed indirect hemolytic activity. The myotoxin CV-PL-F₄ caused a rapid necrosis of skeletal muscles after 1,3 and 24 hours of injection. In addition, CV-PL-F₄ has a phospholipase activity when tested on egg yolk phospholipids. The PLA₂ myotoxin of *Cerastes vipera* venom is similar to other myotoxin isolated from different snake venoms [22,31-34]. This myotoxin has no neurotoxic symptoms or lethal activity in mice injected with 30 µg/25 g. Examination of CV-PL-F₄ using SDS-PAGE electrophoresis revealed that the fraction appeared to be almost pure. The myotoxin CV-PL-F₄ has M.W of about 14,600. Phospholipases A₂ molecular mass range from 13 to 19 kDa [35] and typically requires Ca²⁺ at for their catalytic activity [36]. PLA₂ isolated from snake venoms are much less lethal than neurotoxins [17,37-39]. Some of these toxins exhibited myotoxic activity *in vivo* [38,40-43]. Some of the myotoxic PLA₂ have high enzymatic activity, whereas others lack or have an extremely low catalytic activity. However, a myotoxin was isolated by Gutierrez et al. from the venom of the snake; *Bothrops nummifer* with M.W of 16,000 which lacked PLA₂ activity and induced skeletal muscle damage [44]. On the other hand, Diaz et al. isolated a myotoxic protein fraction from the venom of *Bothrops asper* snake with PLA₂ activity and induced acute muscle damage in mice [32]. This indicates that myotoxins may have or have no PLA₂ activity. The myonecrosis induced by the crude venom and CV-PL-F₄ may be a result of the progressive loss of integrity of the sarcolemma due to the hydrolysis of phospholipids. This is supported by the hemolytic effect of the venom and CV-PL-F₄ on rabbit RBC membrane *in vitro*. Also, the interaction of the fraction with muscle

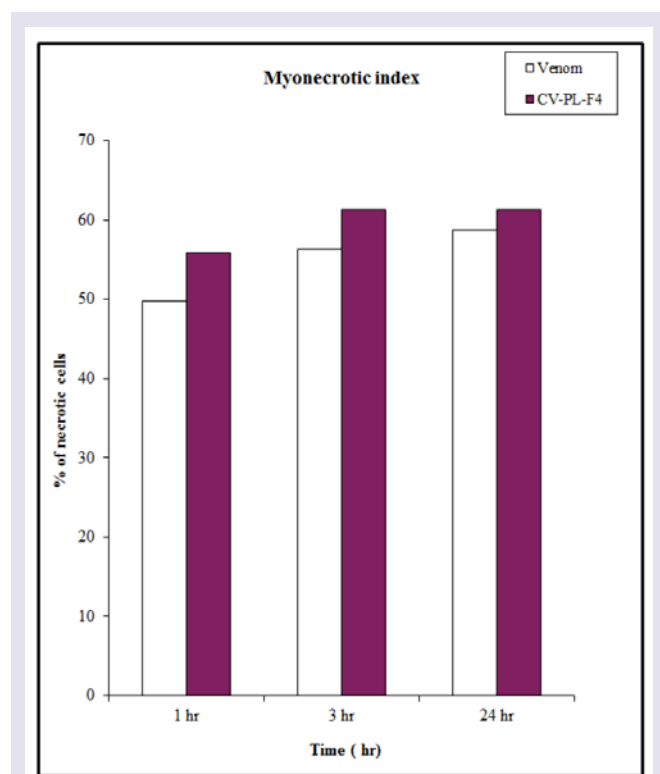


Figure 6: Percentage of myonecrotic cells induced by *Cerastes vipera* and CV-PL-F₄ myotoxin.

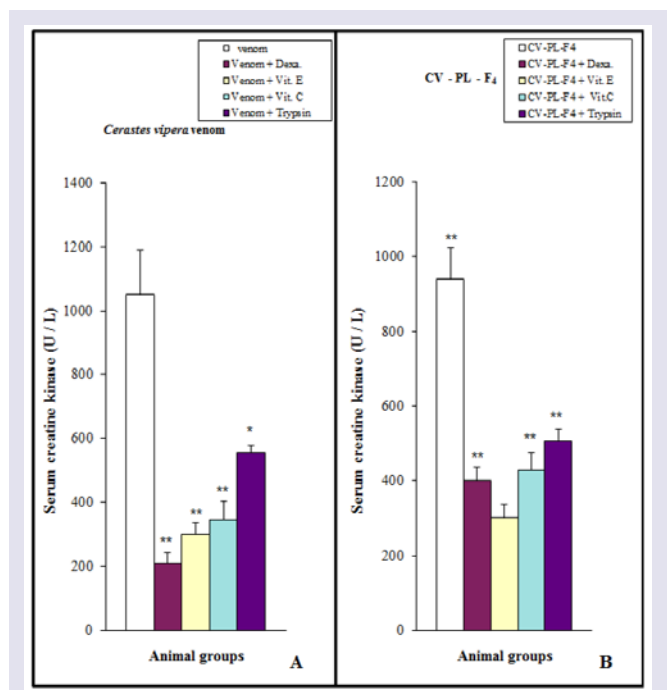


Figure 7: Effect of dexamethasone (Dexta) and vitamins E (Vit. E) and C (Vit. C) and trypsin on creatine kinase release after the injection of *Cerastes vipera* venom (A) and CV-PL-F₄ myotoxin (B). Significant differences were compared to the value of venom- or CV-PL-F₄-treated mice alone in case of (A) and (B). Each value represents mean \pm S.E., n = 8; * P < 0.05; ** P < 0.01.

membrane integrity induced muscle damage mediated by a number of events such as calcium influx and efflux of cytosolic molecules [45,46]. Snake venoms belonging to Viperidae are known to possess a high content of proteinase [47]. *Cerastes vipera* venom (after 3 hr) and CV-PL-F₄ (after 24 hr) induced highest CK release in serum and resulted in extensive muscle tissue damage. Huanchahuire-Vega et al. recorded a high CK level 3- 6 hr after i.m. injection of *Bothrops asper* venom in mice [48]. Tissue damage shows an increase of CK levels in serum since this enzyme is abundant in the cytosol of skeletal muscle fibers [49]. This finding suggests that the plasmatic membrane of these cells has rapidly lost its ability to regulate the permeability to this enzyme [50]. Such alterations are similar to those generated by venom that results in muscular necrosis [37,51]. The results suggest a correlation between the serum levels of CK and the myonecrosis as demonstrated by the myonecrotic index. These findings are similar to other studies on the myotoxic effects of *Bothrops* venoms [31] and South American rattlesnake; *Crotalus durissus terrificus* by Gopalakrishnakone et al. and Azevedo-Marques et al. [52,53]. The degenerative change in the structure of murine skeletal muscle induced by i.m. injection of phospholipase A₂ fraction isolated from *Crotalus durissus terrificus* which was attributed to the hydrolysis of sarcolemma membrane [54]. The myonecrosis observed in the present study was also supported by higher serum creatinine, which resulted from the conversion of creatine [55]. CV-PL-F₄ did not produce visible myoglobinuria as previously observed in other snake myotoxins [38,52,56,57]. Anderson and Ownby observed severe hemorrhage and necrosis of some skeletal muscle cells in mice i.m. injected with a proteinase H fraction isolated from eastern

diamondback rattlesnake (*Crotalus adamonteus*) [58]. Moreover, there is a direct correlation between serum CK release and muscle damage (myonecrotic index) caused by the *Cerastes vipera* venom and CV-PL-F₄ myotoxin ($r = 0.77$ and 0.99 respectively). Clinical studies of myonecrosis, in severe cases of envenoming, have described a generally limited effectiveness of antivenom serotherapy in preventing the development of local tissue damage [59]. Moreover, Gay et al. demonstrated that the hemorrhagic activity of the viper *Bothrops alternatus* was neutralized by anti-baltergin IgG antibodies, but the myonecrotic activity was not ameliorated by this antiserum [15]. Therefore, the use of dexamethasone, Vit E and C as a treatment for envenomation may be more effective than serotherapy. It was found that the myotoxicity of the crude venom and CV-PL-F₄ myotoxin was completely inhibited by dexamethasone. The present results suggested that dexamethasone with its anti-inflammatory property may ameliorate the muscle damage resulted from crude venom and CV-PL-F₄ myotoxin. This suggestion was supported by the decreased CK of the present study. These findings are concomitant with the study of Patrão -Neto et al. showing that dexamethasone ameliorated the inflammation and myotoxicity induced by *Bothrops* venom [60]. Also, Vit E and C reduced the CK released to approximately the control levels in mice treated with the crude venom and CV-PL-F₄. Dexamethasone, Vit E and C and trypsin reduced the myonecrotic index induced by the crude venom and CV-PL-F₄. The mode of action of myotoxic PLA₂ has been thoroughly discussed by Gutiérrez et al. [56]. There exists some evidence that the enzymatic properties of these myotoxins are involved in the necrosis. It may be due to the release of lysolecithin *in vivo* [34,48,56] and hydrolysis of phospholipids in muscle cells. This explanation is supported by the study of the effect of the venom and CV-PL-F₄ myotoxin on the integrity of the cell membrane of rabbit erythrocytes. *Cerastes vipera*

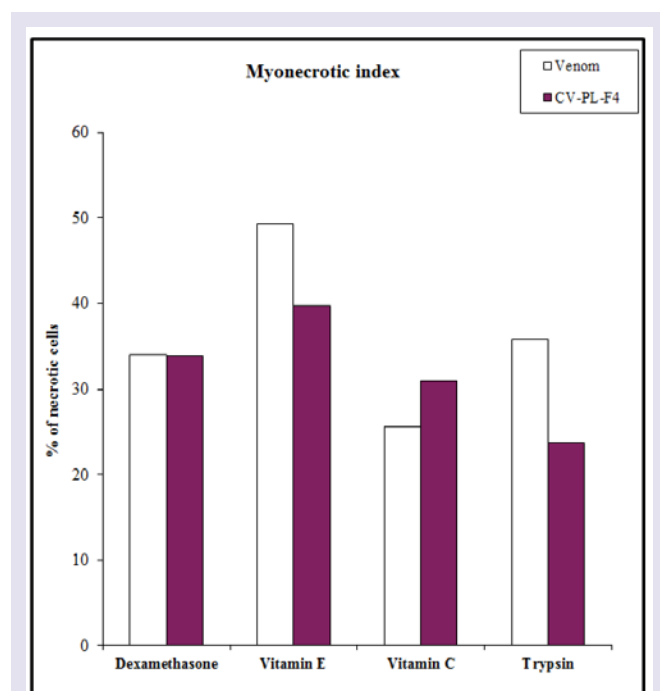


Figure 8: Antagonism of the myonecrosis induced by *Cerastes vipera* venom and CV-PL-F₄ myotoxin using dexamethasone, vitamins E and C and trypsin.

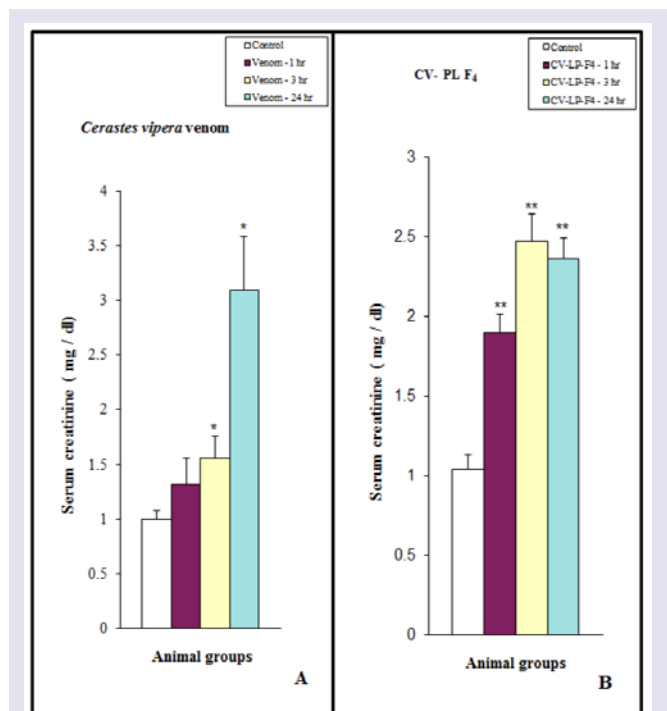


Figure 9: Effect of *Cerastes vipera* venom (A) and CV-PL-F₄ myotoxin (B) on serum creatinine in mice. Each value represents mean \pm S.E., n = 8 * P < 0.05; ** P < 0.01.

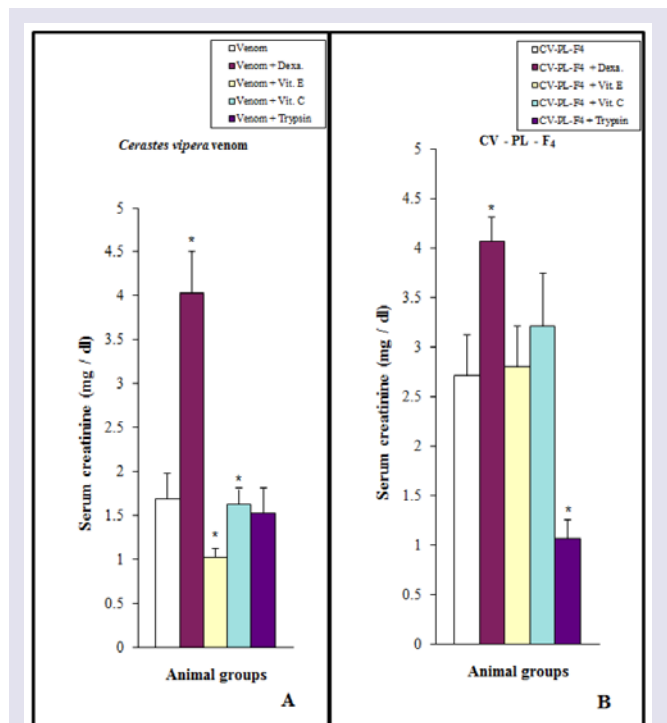


Figure 10: Effect of dexamethasone (Dexa) and vitamins E (Vit. E) and C (Vit. C) and trypsin on serum creatinine after the injection of *Cerastes vipera* venom (A) and CV-PL-F₄ myotoxin (B).

Significant differences were compared to the value of venom-or CV-PL-F₄-treated mice alone in case of (A) and (B) Each value represents mean \pm S.E., n = 8 * P < 0.05.

and its myotoxin hydrolyzed the plasma membrane of rabbit RBCs *in vitro*. This assumption is concomitant with the interpretation of Harris and MacDonnell and Gutiérrez et al. [56,61]. These authors showed alterations of plasma membrane integrity and a general increase in membrane permeability, causing an influx of ions such as calcium, which may also trigger intrinsic mechanisms leading to cell death and necrotic events. The hemolytic activity of the venom of the viper; *Cerastes vipera* and the isolated myotoxin (CV-PL-F₄) appear to be dependent on their phospholipase A₂ activity as recorded in several studies of other snake venoms [2,62,63]. Vit E, as an antioxidant present in membranes, confers stability to the membrane [64,65]. Recently, it was reported the stabilization of lysosomal membranes by Vit E against damage may be inflicted by viper venom PLA₂ [66]. Thus, Vit E partially inhibited the lysis of the plasma membrane and fatty acid release (phospholipid splitting) caused by snake venoms. It seems reasonable to conclude that tocopherol protects cell membrane against the hydrolytic action of the viper venoms. Lysolecithins, products of exogenous phospholipid hydrolysis, cause considerable damage to the cell membranes by venoms and PLA₂. This interpretation was supported by the assessment of the indirect hemolytic activity of the viper venom and its CV-PL-F₄. Jurak and Conde suggested that α -tocopherol plays a role in the stabilization of biological membranes. In spite of dexamethasone and Vit E decreased significantly the CK released from the envenomated muscle, with either crude *Cerastes vipera* or CV-PL-F₄, Vit E was more effective as antimyotoxins [67]. Therefore, regular intake of Vit E might be useful for people living in an area where snakes abound. The crude venom and CV-PL-F₄ myotoxin had proteases activity that may be responsible for the myonecrosis observed in this study. Many snake venoms especially belonging to the vipers and their toxins cause myotoxicity which has been attributed to the presence of proteases or phospholipase A₂ [33,44]. These enzymes caused disruption directly the plasma membrane integrity of the muscle cells [46,68]. It is possible that the myonecrosis may be caused by the development of ischemia resulting from microvascular alteration of muscle tissue by the viper venom and CV-PL-F₄. Such activity has been previously demonstrated by the action of other viper venoms, which have been shown to contain hemorrhagins in their composition [37]. Hemorrhage was observed on the inner surface of the skin and on the outer surface of the gastrocnemius muscle, in addition to the hemorrhage in the lungs. Myonecrosis appeared only after massive intramuscular hemorrhage due to snake venom injection [69]. Estimation of serum levels of CK together with the histological myonecrosis index, in the present study, may be a real evidence of the necrosis of the muscles following envenomation with crude venom and CV-PL-F₄ myotoxin (Figure 10). This is because the quantitative assessment of muscle damage by the estimation of the serum levels of CK only is not valid. The estimation of serum level of CK and the myonecrotic index could be supportive to each other as an indication of myonecrosis. Moreover, dexamethasone and Vit E have ameliorative effect for the myotoxic effect of the crude *Cerastes vipera* venom and the fraction CV-PL-F₄.

Conclusion

The present study supports the hypothesis that the isolated phospholipase A₂ (CV-PL-F₄) by Sephadex G-75 exhibited high myotoxic activity, demonstrating its capacity to contribute to tissue

damage after snakebite or envenoming produced by this *Cerastes vipera* snake. This topic needs further investigation and will be considered in the future. On the other hand, dexamethasone and Vit E were more effective in alleviating the myotoxic effect of the crude *Cerastes vipera* venom and its fraction (CV-PL-F₂). Therefore, it may be more beneficial if the dose increased in future studies.

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