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Effect of *Cerastes cerastes* snake venom on the neuromuscular transmission of the chick biventer cervicis muscle preparation

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Abstract

Cerastes cerastes is an Egyptian viper from the family Viperidae and genus Cerastes. In general, snakes of the Elapidae family produce neurotoxic venoms targeting the nervous system, while snakes of the Viperidae family produce hemotoxic venoms targeting blood coagulation. Little is known about the neurotoxic action of cerastes venom. The present work was aimed to study the effect of *C. cerastes* crude venom on neuromuscular transmission, and to isolate and purify the fraction(s) responsible for that neurotoxicity. The venom was fractionated by gel filtration on sephadex G-50. The neurotoxicity was verified by neuromuscular technique (chick biventer cervicis muscle preparation, CBCM). Five different fractions (F1, F2, F3, F4, and F5) were collected by gel filtration. Both crude venom (40 μg/ml) and F1 (20 μg/ml) exhibited obvious neurotoxicity. They significantly reduced the twitch tension of (CBCM) and suppressed the contractile responses of CBCM to ACh (10^{-3} M), carbachol (CCh, $2x10^{-6}$ M), and KCl (60 mM). These results suggest that crude venom and F1 affect peripherally, and they are postsynaptically acting. Crude venom has phospholipase and proteinase activities, while the neurotoxic fraction (F1) has proteinase activity only.

Key words: cerastes venom; chick biventer cervicis muscle preparation; phospholipase A2; gel filtration.

1. Introduction:

In many countries, snake envenomation is a major health concern **[1]**. The World Health Organization reports that there are over 5 million snake bites annually, with over 100,000 mortality cases **[2].** Snake venoms are rich sources of pharmacologically active polypeptides and proteins. Some of these proteins exhibit enzymatic activities. These enzymes include phospholipase A2, proteinase, nucleotidase, phosphodiesterase, and L-amino acid oxidase **[3-4].** In addition to their catalytic properties, these enzymes induce also various pharmacological effects including neurotoxic, myotoxic, cardiotoxic, hemorrhagic, hemolytic, procoagulytic, and anticoagulytic effects **[5-6].** Other snake

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venom proteins do not exhibit these enzymatic activities and thus are described as nonenzymatic proteins. These proteins include neurotoxins, cardiotoxins, myotoxins, ion channel inhibitors, and anticoagulant proteins **[7].** Thus, snake venom proteins, whether enzymatic or nonenzymatic, target several tissues, organs, and physiological systems and interfere with their normal functions.

The World Health Organization (WHO) considers about 300 species, mainly belonging to the families Viperidae, Elapidae and Colubridae, to be of medical importance **[8].** Vipers of the genus *Cerastes* are the most significant clinically among several hundred venomous snakes. The sand viper *Cerastes cerastes* (horned viper) is widely spread in Egypt, North Africa, and the Arabian Peninsula, where 2750–67,500 snakebites are recorded, leading to 35–62 deaths each year **[9-12].** *C. cerastes* venom is a cocktail of potent molecules that target multiple biologically important systems. The majority of these molecules cause hemolysis, bleeding, respiratory failure, alterations in the coagulation system, metabolic alterations, and variations in the erythrocyte, leukocyte, and platelet counts, all of which frequently result in mortality. The synergistic action of venom components is typically responsible for these harmful effects **[13-14].** There are very few researches that demonstrate if the venom contains neurotoxic elements or affects the nervous system.

Snake venoms primarily target the prey's critical systems, particularly the circulatory and neurological systems. Snakes of the Viperidae family create hemotoxic venoms that primarily affect blood coagulation, whereas snakes of the Elapidae family often produce neurotoxic venoms that contain toxins that target the nervous system. But it's not really evident. The distinction between hemotoxic and neurotoxic venoms is completely arbitrary, as viperid venoms can cause neurological symptoms while elapid venoms can affect blood coagulation **[7,15].** It is now recognized that hemotoxic venoms indeed contain neurotoxic components, and certain viperid bites cause neurotoxic symptoms. Viperid phospholipases A2, for instance, may exhibit pre- or postsynaptic activity and have a role in analgesia and pain. Other neurotoxins that are present in hemotoxic venoms fall into a number of different families, from large multi-subunit proteins (like C-type lectin-like proteins) to small peptide neurotoxins (such waglerins and azemiopsin). Additional neurotoxins generated from hemotoxic venoms include sarafotoxins, crotamine, cysteine-rich secretory proteins, baptides, Kunitz-type protease inhibitors, and three-finger toxins. Some of these toxins exhibit postsynaptic activity, whereas others affect voltage-dependent ion channel function **[16].**

Snake venom's neurotoxicity is primarily verified by disruption of neuromuscular transmission, notably in the skeletal muscles **[17].** Venomous snakes are predators without claws or strong paws, but since their victims' skeletal muscles are paralyzed, they do not need these features because their prey is unable to breathe, flee, or resist **[18].** The following components of the mechanism of electrochemical impulse transmission from nerve to muscle may be the targets of the majority of snake venom neurotoxins: sodium and potassium channels of the nerve fiber; the release of the mediator from the pre-synaptic membrane; the passage of the released mediator in the synaptic cleft; the receptors on the post-synaptic membrane of the muscle receiving the mediator; and the sodium, potassium, and calcium channels of the muscle fiber. Each neurotoxin often only affects one of these targets due to the selectivity of snake venom poisons. While viperid neurotoxins are less common and only affect a few of targets, the neurotoxins found in elapid venoms affect almost all of these targets **[16,18].** Even though viperid venoms are known to have neurotoxic effects, but the reviews on viperid neurotoxins are rare. This study explores whether *C. cerastes* venom, known to be hemotoxic, can be neurotoxic and can affect the peripheral nervous system.

Aim of work

The present work was aimed to test if the *C. cerastes* crude venom has a neurotoxic effect. To study the effect of the neurotoxic fraction(s) on the neuromuscular transmission and to determine if the toxin affects presynaptically or postsynaptically.

2. Materials and Methods:

2.1. Reagents and venom.

Acetylcholine chloride, carbachol, potassium chloride, lecithin and sephadex-G50 were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All salts for the physiological solutions and buffers were of analytical grade. *Cerastes cerastes* venom was extracted from adult vipers collected from Toushka region at Aswan, Egypt. They were kept at the serpentarium of the Zoology Department, Faculty of Science, Ain Shams University. The venom was obtained from the vipers by allowing them to bite naturally through a layer of rubber covering a glass beaker. The venom was then lyophilized (Labconco lyophilizer, USA) and stored desiccated at room temperature in a dark vial until used.

2.2. Fractionation of *Cerastes cerastes* venom

According to Batzri-Israel and Bdolah **[19]**, samples of lyophilized *Cerastes cerastes* venom (100 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-50 equilibrated with the same buffer. The elution was at a flow rate of 18 ml per hour using an EP-1 Econo Pump (Bio- Rad Laboratory, Hercules, CA, USA). Five-millilitre fractions were collected by an automatic fraction collector (Pharmacia, Sweden). The total protein content of each tube was determined by absorbance measurements at 280nm (A_{280}). The resulting optical densities were drawn against the elution volume on millimeter paper. Tubes representing each fraction were pooled together then lyophilized.

2.3. *In vitro* Neuromuscular Preparations (Chick biventer cervicis muscle preparation).

Male chicks (3 - 12 days old) were killed by CO_2 inhalation and both biventer cervicis muscles were dissected by the method of Ginsborg and Warriner, **[20]**. The muscles were mounted under a tension of 1 gm in a 10 ml organ bath containing physiological Krebs solution of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. This solution was maintained at 34°C and bubbled with carbogen (95% O_2 and 5% CO_2). Submaximal contractures to exogenously applied acetylcholine (ACh; 10^{-3}M for 30s), Carbachol (CCh; $2x10^{-6}$ M for 60s) and potassium chloride (KCl; 60 mM for 30s) were obtained in the absence of electrical field stimulation prior to addition of venoms and at the end of the experiment. According to Harvey, **[21]**, the preparations were stimulated with a bipolar platinum ring electrode coupled to a Grass S88 stimulator, USA. Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1Hz. Isometric muscle contractions and contractures were recorded via a force displacement transducer (Grass Model 79 polygraph transducer, USA) with chart speed 5 mm/sec. The preparations were allowed to stabilize for at least 20 min before the addition of drugs or venom.

2.4. Determination of venom phospholipase A_2 activity:

Crude venom and each of the lyophilized fractions were assayed for phospholipase A_2 activity according to the method of Augustyn and Elliot **[22].** The method is based on the reaction between hydroxylamine hydrochloride in alkaline medium with the ester linkage producing a brown color. The decrease in the developed colour due to hydrolysis of ester linkage by the enzyme was taken as an index for enzyme activity. The phospholipase A_2 activity was calculated in µg lecithin hydrolysed /h /mg protein from the standard curve.

2.5. Determination of venom acetyl cholinesterase (AChE) activity.

Crude venom and each of the lyophilized fractions were estimated using the colorimetric method of Ellman *et al*. **[23],** as modified by Gorun *et al.* **[24].** The cholinesterase of the venom was incubated with acetylthiocholine iodide 5mM (Ubichem Limited, Germany) for specific time interval and the reaction was then stopped with a reagent of 5.5´dithiobis-2-nitrobenzoic acid (DTNB) for colour development. The reaction of thiocholine, resulting from enzymatic hydrolysis of acetylthiocholines with DTNB is pH dependent (not below pH 6.5 and not higher than pH 8). The optical density of yellow color was read immediately at 412 nm and the level of AChE activity was obtained from a standard curve.

2.6. Determination of venom protease activity.

Protease activity was estimated by the ultraviolet method of Labib *et al*. **[25].**

2.7. Statistical analysis / graph plotting.

Statistical comparisons were made using student's t-test and one-way ANOVA with repeated measures using the Minitab Statistical Software Package. The differences were considered significant at $P<0.05$. Data were graphed using graphpad prism version 4 and Origin version 6.1.

3. Results:

For the fractionation of the *C. cerastes* venom, Sephadex G-50 was used using 0.1 M ammonium acetate buffer, pH 6.8 and column dimensions 2.6 x 74 cm. at a flow rate of 18 ml /hr. As shown in table1 and fig.1, the venom was separated into five fractions (five peaks).

Table 1. Fractionation of *C.* crude venom on Sephadex G-50 using 0.1 M ammonium acetate buffer solution, pH 6.8 (column dimensions 2.6 × 74 cm) at a flow rate 18 ml/hr and elution volume 4 ml/tube.

Elution Vol.	Abs	Elution vol.	Abs	Elution Vol.	Abs	Elution vol.	Abs	Fraction No.	Elution vol.
4	0	84	0.6	164	0.33	244	0.04	F1	8- 64
8	0.002	88	0.54	168	0.26	248	0.03	F2	68- 100
12	0.1	92	0.48	172	0.2	252	0.02	F3	104- 132
16	0.37	96	0.4	176	0.12	256	0.01	F4	136- 147
20	0.705	100	0.35	180	0.08	260	0	F5	212- 256
24	0.88	104	0.32	184	0.055	264	0		
28	1	108	0.42	188	0.04	268	0		
32	1.11	112	0.47	192	0.01	272	0		
36	1.22	116	0.49	196	0	276	0		
40	1.32	120	0.5	200	0	280	0		
44	1.39	124	0.48	204	0				
48	1.28	128	0.45	208	0				
52	1.08	132	0.34	212	0.025				
56	0.94	136	0.29	216	0.065				
60	0.66	140	0.43	220	0.12				
64	0.55	144	0.5	224	0.16				
68	0.53	148	0.52	228	0.16				
72	0.57	152	0.51	232	0.15				
76	0.61	156	0.45	236	0.14				
80	0.62	160	0.37	240	0.08				

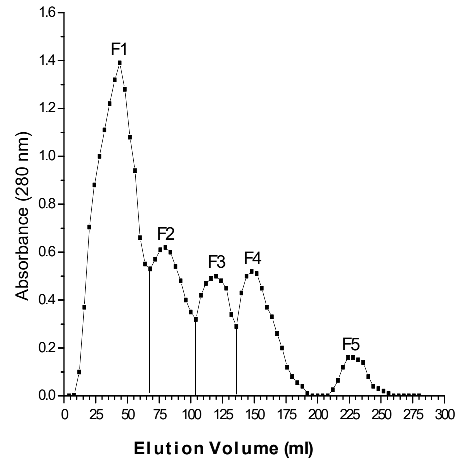

Fig.1. Fractionation of C cerastes crud venom using sephadex G-50

2. *In vitro* Neuromuscular Preparations (Chick biventer cervicis muscle preparation).

The crude *C.* c*erastes* venom and its five gel filtration fractions were tested for their ability to affect neuromuscular transmission by testing their effect on the contractile response of the CBCM evoked by nerve stimulation. The results showed that the crude venom produced a concentration dependent and progressive decrease in the twitch response of the CBCM. As shown in Fig. 2 and 3, 10 µg/ml and 20 µg/ml had no effect on the twitches. However, 40 µg/ml of venom was effective dose and caused more than 70% inhibition of the original twitch height within about 120 min. In addition, the crude venom significantly inhibited contractile responses to the exogenous nicotinic agonists (i.e. ACh and CCh; $p<0.05$). It inhibited the response to ACh (10^{-3} M) to about 95.3 ± 2.1% and to exogenous CCh ($2x10^{-6}$ M) to about 94.9 ± 2.5% of the control. Also, the venom significantly ($p<0.05$) reduced the response to exogenous KCl (60 mM) to about 78.3 ± 2.6% of the control (Fig.3). Washing of the preparation after inhibition of the twitches did not result in any significant recovery in the twitch height.

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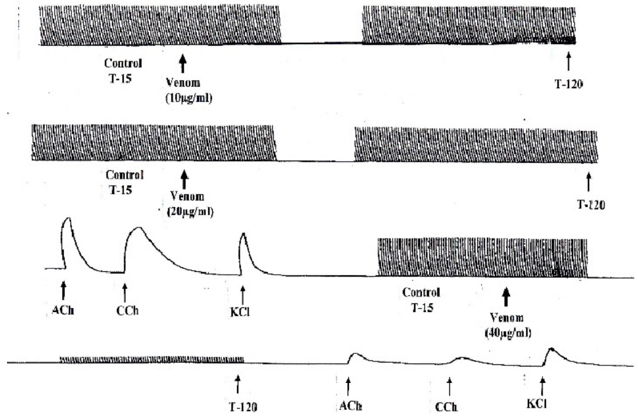
Fig. 2. Typical tracings showing the effects of the venom of *C. cerastes* on twitch responses of the CBCM evoked via nerve stimulation (a) 10 µg/ml, (b) 20 µg/ml, (c) responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 40 µg/ml venom. T, time (min).



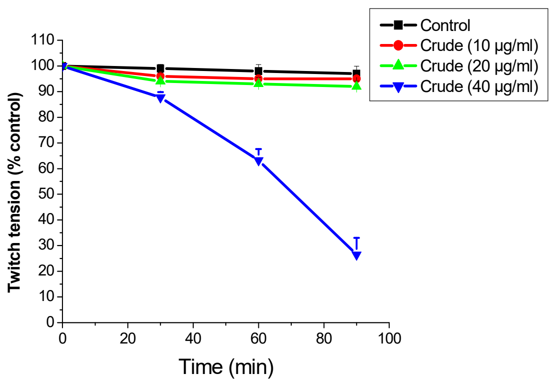


Fig 3. Effects of *C. cerastes* crude venom (10, 20 and 40 µg/ml) on twitch of CBCM. Each point represents the mean of values obtained from four experiments
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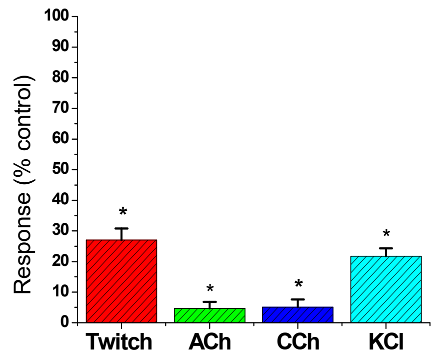
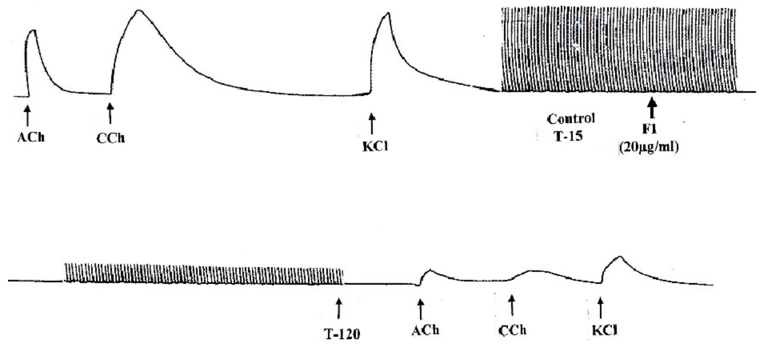


Fig. 4. Effects of *C. .cerastes* crude venom (40 µg/ml) on responses of CBCM (twitch), ACh, CCh and KCl Data are presented as means ± SEM of four experiments. * $P<0.05$

Similarly, twitch response of the chick muscle preparation was reduced in the presence of F1 in a time and concentration dependent manner. F1 at 20 µg/ml inhibited the twitches by about 23.8 ± 12.2%, 42.5 ± 14% and 76.7 ± 7.3% of the control in about 40, 80 and120 min respectively (Fig. 4 and 5). As shown in Fig. 6, F1 significantly ($p<0.05$) reduced contractile response to exogenous nicotinic agonists ACh (10^{-3} M) and CCh ($2x10^{-6}$ M) to about 73.5 ± 5.6% and 82.4 ± 4.7% respectively. The fraction also reduced the response to exogenous KCl (60 mM), this reduction was significant ($P<0.05$) and reached about 58.8 ± 3.8% of the control.

Fig. 5. Typical tracings showing the effects of F1 (20 µg/ml) of the *C cerastes* crude venom on twitch responses of the CBCM evoked via nerve stimulation. Responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 20 µg/ml F1. T, time (min).



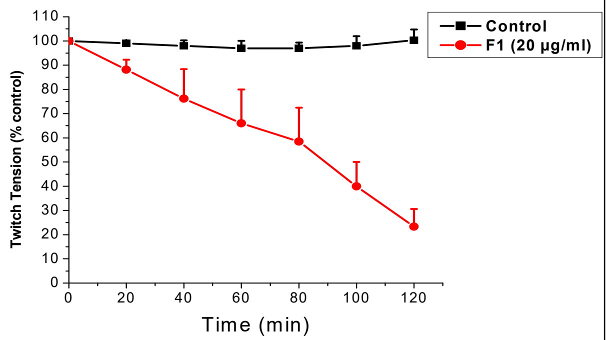


Fig 6. Effects of F1 of *C. cerastes* venom 20 µg/ml on twitch of CBCM. Each point represents the mean of values obtained from four experiments

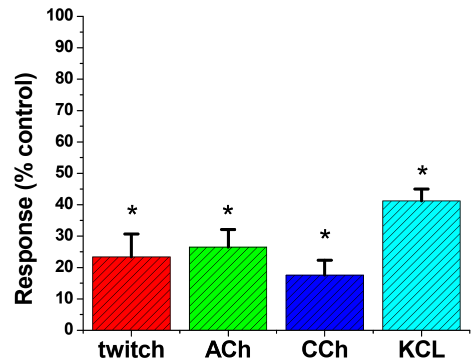


Fig. 7. Effects of *C. cerastes* venom (20 µg/ml) on responses of CBCM (twitch), ACh, CCh and KCl Data are presented as means ± SEM of four experiments. * P<0.05

From fig. 8, 9, 10, and 11, it was obvious that the other fractions (F2, F3, F4 and F5) did not show any significant change in the twitch response of the CBCM. 100 µg/ml of F2 had no effect on the twitch tension after 120 min compared with the control. Similarly, F3 (100 µg/ml) did not record any significant change in the twitch, only a slight and insignificant decrease to about 7.6 ± 0.8% of that of the control after 120 min. On the contrary, a slight and insignificant increase in the contractile response after exposure to F4 (100 µg/ml), the twitch response increased to about 5.1 ± 0.7% of that of the control after 120 min. F5 was similar to F4; it caused an increase in the contractile response of the muscle. This increase reached about 13.5 ± 6.2% of the control, but statistically this increase was not significant when compared with that of the control.

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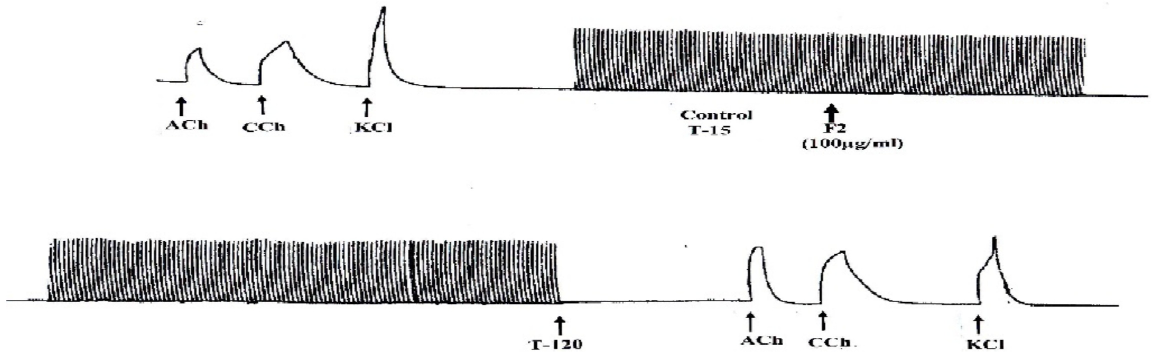

Fig. 8. Typical tracings showing the effects of F2 (100 µg/ml) of the *C cerastes* crude venom on twitch responses of the CBCM evoked via nerve stimulation. Responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 20 µg/ml F1. T, time (min)

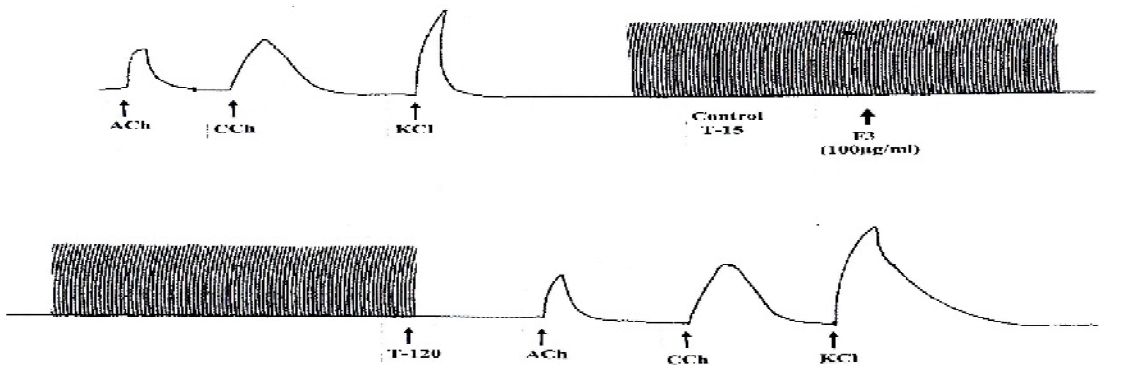

Fig. 9. Typical tracings showing the effects of F3 (100 µg/ml) of the *C cerastes* crude venom on twitch responses of the CBCM evoked via nerve stimulation. Responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 20 µg/ml F1. T, time (min)

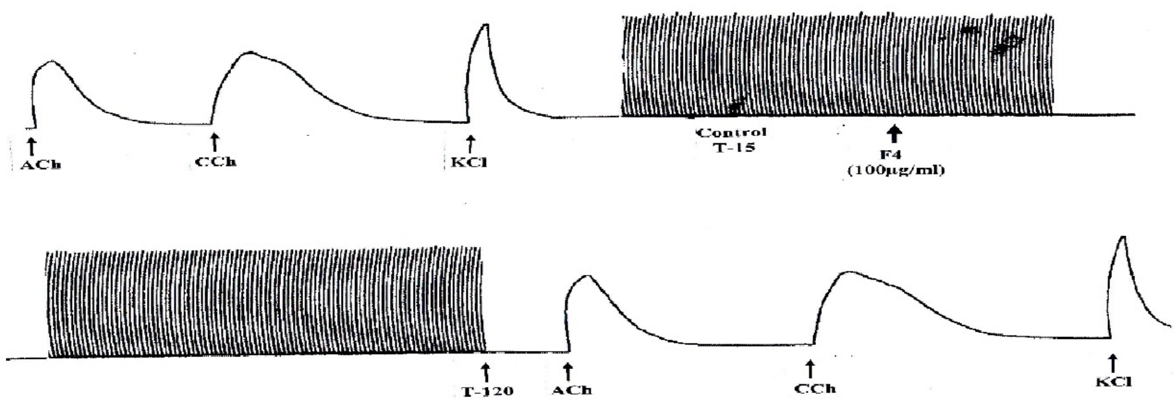

Fig. 10. Typical tracings showing the effects of F4 (100 µg/ml) of the *C cerastes* crude venom on twitch responses of the CBCM evoked via nerve stimulation. Responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 20 µg/ml F1. T, time (min)

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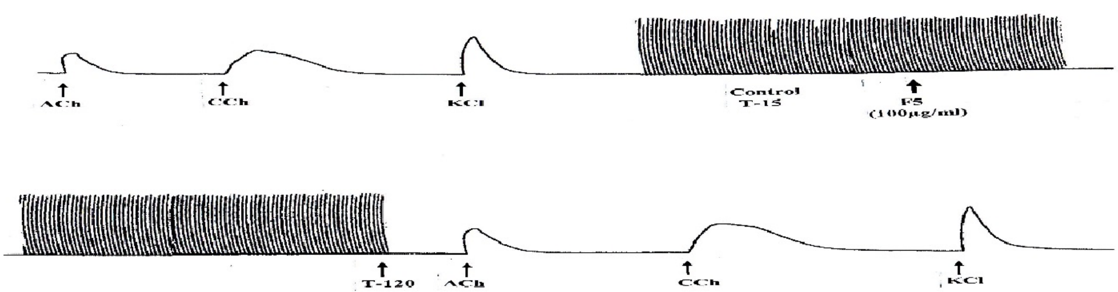

Fig. 11. Typical tracings showing the effects of F5 (100 µg/ml) of the *C cerastes* crude venom on twitch responses of the CBCM evoked via nerve stimulation. Responses to ACh (10^{-3} M for 30 sec), CCh ($2x10^{-6}$ M for 60 sec) and KCl (60 mM for 30 sec) before and and after addition of 20 µg/ml F1. T, time (min)

Fig. 13 showed the effects of fractions 2, 3, 4, and 5 on the contractile response to the exogenous nicotinic agonists ACh (10^{-3} M) and CCh ($2x10^{-6}$ M) and to KCl (60 mM). The contractile response to ACh and CCh was increased after 120 min of F2 exposure; this increase was insignificant and reached levels of about 15 ± 1.5% and 11.4 ± 0.8% of the control respectively. F2 decreased the contractile response of KCl to about 12 ± 0.8%, but this decrease was also not statistically significant. Similarly, F3 had no significant effect on the contractile response to ACh, CCh and KCl. Contractions were decreased to about 3.4 ± 1.6% and 3.4 ± 0.6% of the control after ACh and KCl addition respectively and a slight insignificant increase (2.5 ± 12.5%) of the control after CCh.

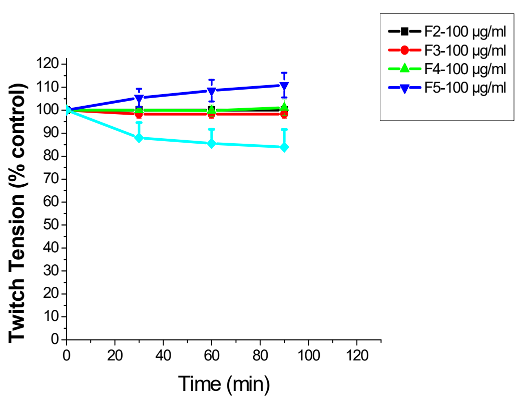

Fig. 12. Effects of F2, F3, F4, and F5 of *C. cerastes* crude venom (100 µg/ml) on responses of CBCM to nerve stimulation. Each point represents the mean of values obtained from four experiments and SEM is indicated by the bars unless these bars are smaller than symbols.

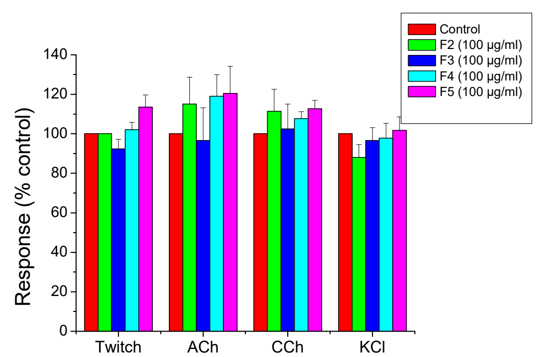

Fig. 13. Effects of fractions 2, 3, 4, and 5 of *C. cerastes* crude venom (100 µg/ml) on responses of CBCM preparations to nerve stimulation (twitch), ACh, CCh and KCl at the following concentration: fraction 100µg/ml, ACh 10^{-3} M, CCh $2x10^{-6}$ M and KCl 60 mM. Data are presented as means ± SEM of four experiments.

F4 did not cause any significant change in the responses to ACh, CCh and KCl. The fraction increased slightly the contractile response to ACh and CCh. In case of ACh, the increase reached about 19 ± 1.9% of the control, while in case of CCh the increase was

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about 7.7 ± 1.6% of the control. The fraction decreased the response to KCl to about 2.2 ± 0.5% of the control (Fig. 13). Similarly, there was an increase in the contractile response to ACh and CCh following F5 application. The increase was insignificant and reached about 20.4 ± 3.7% and 12.7 ± 1.2% of the control respectively.

Enzymatic activity of crude venom and its fractions

1) Phospholipase A_2

As shown in table 2 and fig. 14, phospholipase activity was recorded in the crude venom and some of its fractions. The crude venom recorded PLA_2 activity of about 400 ± 25 µg hydrolyzed lecithin / hr/mg protein. The activity was varied in the fractions. F2 recorded the highest activity of about 760 ± 44 µg hydrolyzed lecithin / hr/mg protein, while PLA_2 activity of F4 was 660 ± 33 µg hydrolyzed lecithin / hr/mg protein. On the contrary, fractions 1, 3, and 5 did not show any phospholipase activity.

Table 2. Determination of phospholipase A_2 (PLA_2) activity (µg hydrolyzed lecithin /hr/mg protein) in *C. cerastes* crude venom and its six gel filtration fractions.

Sample	Activity µg lecithin hydrolyzed/hr/mg protein
Crude *C. cerastes*	400 ± 25
F1	-ve
F2	760 ± 44
F3	-ve
F4	660 ± 33
F5	-ve

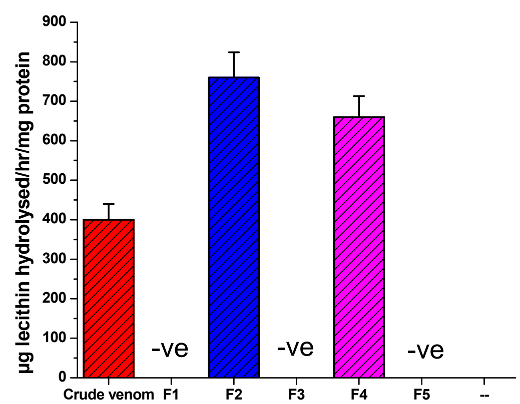

Fig. 14. Phospholipase A_2 activity of C..*cerastes* crude venom and its five gel filtration fractions. Data are expressed as means ± SEM for four tests.

2) Proteinase:

The data presented in table 3 and fig. 15 revealed the presence of proteolytic activity in the crude venom and some of its fractions. This activity was pronounced in fractions 1, 3 and 6 while other fractions lack this activity.

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Table 2. Determination of proteinase activity (caseinolytic activity) in *C. cerastes* crude venom and its five gel filtration fractions.

Sample	Specific activity (ΔA_{280} /hr/ mg)
Crude *C.cerastes*	2.03 ± 0.3
F1	2.9 ± 0.26
F2	-ve
F3	1.71 ± 0.26
F4	-ve
F5	-ve

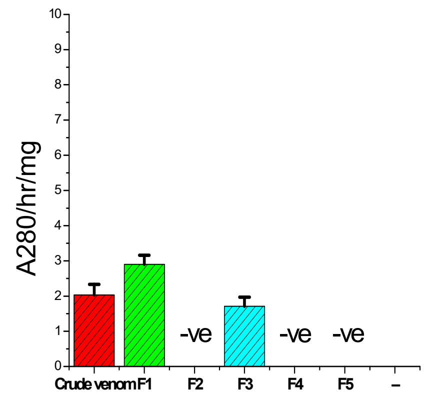

Fig. 15. Proteinase activity in crude *C. cerastes* venom and its five fractions. Data are expressed as means ± SEM for four values.

3) Acetylcholinesterase (AChE):

C. cerastes crude venom and all of the five fractions didn't show any AChE activity. This result was expected as many viperid venoms lack such activity venoms.

4. Discussion

Snake venoms are complex mixtures of pharmacologically active protein and polypeptide toxins. A large number of these toxins can interfere with cholinergic transmission at postsynaptic or presynaptic sites of the neuromuscular junction **[26-27].** Postsynaptic neurotoxins bind to postsynaptic nicotinic acetylcholine receptors (nAChR) and interfere with the neuromuscular transmission. These toxins display different binding kinetics and different affinity for subtypes of nicotinic receptors. Post-synaptic neurotoxins are mostly identified in venoms from the families Elapidae and Hydrophiidae **[7,28-30].** Most pre-synaptic toxins are either phospholipase A_2 enzymes or contain these enzymes as an integral part of the neurotoxin complex and mediate their neurotoxicity by inhibiting the release of acetylcholine **[31-33]**

Hemotoxic venom of *Cerastes cerastes* viper affects the functions of the central nervous system by disturbing the platelet aggregation and blood clotting system, as well as by damaging vascular endothelium which produces severe intracranial bleeding **[33].** Mostly, these effects will be accompanied by severe neurological disorders. Such complications are attributable to venom metaloproteinases **[34],** serine proteinases **[35],** and disintegrins. Should these compounds be considered as neurotoxins? Apparently, they

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should not, as they don't target any particular excitable tissues. Disruption of neuromuscular transmission, notably in the skeletal muscles, is the primary manifestation of snake venom's neurotoxicity.

The chick biventer cervicis nerve muscle (CBCM) preparation is a suitably useful screening tool for the examination of animal venoms and toxins **[21].** Unlike the rat or mouse phrenic nerve–hemidiaphragm preparations, the avian preparation contains both multiply and focally-innervated muscle fibers. The former mediates the electrically evoked twitch; whereas the latter can be stimulated and generate a contracture on exposure to exogenous nicotinic agonists, such as carbachol or ACh. This property allows the distinction between pre- and post- synaptic activity. **[21, 36- 38].**

The crude *C. cerastes* venom and its five gel filtration fractions were tested for their ability to affect neuromuscular transmission. (their effects on the contractile response of the CBCM evoked by nerve stimulation). Using an avian neuromuscular preparation, the present results have shown that the crude venom produced a concentration dependent and progressive decrease in the twitch response of the CBCM. No significant effect was recorded with the small doses (10 µg/ml and 20 µg/ml) on the twitch of muscle. However, 40 µg/ml of venom was an effective dose and caused more than 70% inhibition of the original twitch height. In addition, the crude venom significantly inhibited contractile responses to the exogenous nicotinic agonists (i.e. ACh and CCh; $p<0.05$). Furthermore, the venom significantly ($p<0.05$) reduced the response to exogenous KCl (60mM) compared to the control. Washing of the preparation after inhibition of the twitches did not result in any significant recovery of the twitch height.

In the present work, the crude venom caused suppression of the contractile responses of CBCM to exogenous ACh and CCh (both of which produce agonistic responses in skeletal muscle via interaction with postsynaptic nAChRs) suggesting a postsynaptic site of action. This appears to be pharmacologically similar to that demonstrated by elapid venom α-neurotoxins **[39-40].** Postsynaptic neurotoxins bind to nicotinic ACh receptors with a high affinity and competitively antagonize the actions of ACh and CCh **[27,32,37].** However, responses to exogenous KCl were inhibited also indicating that myotoxic activity may contribute to the observed decrease in twitch height. According to **Harvey *et al.* [21],** it is expected that the presence of the myotoxic components in the snake venoms would reduce the contracture response of the skeletal muscle at addition of high concentration of potassium. Normally, the presence of postsynaptic neurotoxins, which have a much quicker onset of action than presynaptic neurotoxins, mask the effects of presynaptic neurotoxins making it difficult to confirm their presence in whole venoms **[37].**

This neuromuscular action apparently involved interference with the neurotransmission as shown by the reduced response to ACh and CCh after incubation with the venom **[21,41,42].** Such inhibition of neurotransmission could involve damage to the nicotinic receptors by proteases present in this venom as well as damaging to muscle fibers. The action on muscle is based on the observation that the venom depressed the responses to K^+ **[26].** These explanations are supported by several reports. **Souza *et al.* [43]** working on *Bothrops jararacuss* venom, **[44]** working on *Bungarus candidus* venom, **[21,41-42]. [37]** working on *Boiga dendrophila* venom and **Kuruppu *et al.* [32]** working on *Demansia papuensis* venom.

The activity of venom may be due to ion channel interaction like that observed for lignocaine, a sodium channel blocker. It reduces twitch height in the electrically stimulated rat vas deferens preparation **[45]** and at higher doses reduces the amplitude of indirectly stimulated twitches and ACh contractures in the CBCM preparation**.** So, the effect of *Cerastes cerastes* venom on muscle twitch may be attributed to its antagonistic effect on Na^+ channel.

Twitch response of the chick muscle preparation was significantly reduced in the presence of F1 of crude venom. At a concentration of 20 µg/ml, the fraction reduced the twitches more than 75% after 120 min. A significant reduction in the contractile response to exogenous ACh (10^{-3} M), CCh ($2x10^{-6}$ M) and KCL (60mM) was remarkable after F1 treatment. Similarly, F1 (10 µg/ml) of *Cerastes cerastes cerastes* caused a significant decrease in the contractile response of the hemidiaphragm muscle to phrenic nerve stimulation **[46]**. As shown from the results, F1 was effective at a small dose (20 µg/ml) compared with 40 µg/ml in crude venom. So, it is concluded that the effectiveness and potency of neurotoxicity increases with fractionation and purification.

On the other hand, the other fractions F2, F3, F4 and F5 lack any effect on the neuromuscular transmission. They did not show any significant change in the twitch response of the CBCM to nerve stimulation. Even with the large dose (100 µg/ml), the fractions have no significant effects on the twitch tension throughout 120 min and did not cause any change compared with the control. Furthermore, no one of these fractions caused a remarkable change in the contractile response to nicotinic agonists (ACh and CCh) or to KCL.

From the above results, it is clear that most of the neurotoxicity of the crude *C.* cerastes venom is concentrated in F1, as it was the only fraction that has an ability to block neuromuscular transmission and nerve conductance. F1 was not a pure fraction, so it was important to be subjected to other purification steps such as Ion exchange chromatography and RP-HPLC.

5. Conclusion

C. cerastes venom showed neurotoxic activity which was evidenced by its effect to block neuromuscular transmission through its effect on CBCM. Five fractions were isolated by gel filtration, the first one has proteinase activity. Both the crude venom and the first fraction act postsynaptically (block the nicotinic receptors) in the neuromuscular junction. Furthermore, they have action on muscle (myotoxic) which is based on the observation that the venom and the fraction depressed the responses to KCl.

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