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Toxicological Effects of the Horned Viper Venom Cerastes Cerastes and Their Neutralization With the Egyptian Scorpion *Androctonus Australis Heamolymph*

KEYWORDS: Cerastes Cerastes venom, Scorpion haemolymph, , Antivenom , Mice, Oxidative stress

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ABSTRACT *The horned viper Cerastes cerastes is a venomous snake belongs to the viperidae family. This study was conducted to examine the toxicological effects of C. cerastes venom in mice and to investigate the neutralizing effects of Androctonus australis scorpion heamolymph. 1/5 LD50 (0.396 mg\kg) of crude venom was injected intraperitoneally and various biochemical parameters have been evaluated at different time intrvals. Our results revealed that oxidative stress biomarkers (lipid perioxidation, plasma protein carbonyl and plasma nitric oxide) as well as plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased while, blood and liver glutathione and plasma total protein (PTP) were significantly decreased at 2, 4, 16 and 24 hours post venom injection. Interestingly, the haemolymph of the Egyptian scorpion A. australis neutralized the toxic effects of snake venom with the same efficacy of commercial polyvalent viper's antivenom. We conclude that the sera of Egyptian scorpions may have certain antitoxic proteins able to neutralize the toxicity of Cerastes cerastes venom.*

Introduction:
Snakebites represent a great medical problem all over the world as they incriminated in a lot of mortalities and morbidities. One of the venomous snakes distributed in Egyptian deserts is the horned viper *Cerastes cerastes* that belongs to the family of viperidae (Underwood, 1979). This species is native to the deserts of Northern Africa and parts of the Middle East, extending through Sinai and Saudi Arabia (Mallow et al., 2003). Snake venom is composed of proteins mainly enzymes in addition to non-protein constituents, which are divided into inorganic and organic components. The majority of snake venoms induce serious changes in one or more body organs of the victim (Masood, 2012). Viper venoms are slowly absorbed and reach maximum serum levels within 6-24 hours (Warrell, 1995). Viperidae toxicity is including cytotoxicity, haemotoxicity and neurotoxicity (Masood, 2012; WHO, 2012).The lethal effects of snake venom are largely attributed to its active ingredient phospholipase A2 (PLA2) which results in the formation of potentially toxic reactive oxygen species (ROS) and lipid peroxides (Neuzil et al., 1998; Adibhatla et al., 2003). PLA2 from snake venoms has been implicated in multiple toxicities including neurotoxicity (Petan et al., 2005), nephrotoxicity (de Castro et al., 2004), lung toxicity (Uma et al., 2000), hepatotoxicity (Mukherjee and Maity, 1998) and cardiotoxicity (Cher et al., 2005). Agents with antioxidant properties have been shown to attenuate viper venom-induced cellular damage by inhibiting the oxidative cascade and improving membrane stabilization (Mukherjee et al., 1998; Alam and Gomes, 1998). The aim of this study was to examine the toxicological effects of the Egyptian snake venom Cerastes cerastes in mice and to evaluate the possible neutralizing efficacy of haemolymph collected from the Egyptian scorpion *Androctonus australis.*

Materials and Methods
Venom collection and preparation
Ten of horned viper snakes were collected from Aswan-Jarf Hussin-Abu steit Mountain (Aswan Governorate, Egypt) then stimulated for milking and collecting venom. The fresh venom was lyophilized and stored at −20 °C till use.

Haemolymph collection and preparation
Fifty of *Androctonus australis* (AA) scorpions were collected from Wadi Natrun (Egypt). Scorpion heamolymph was obtained by aseptic puncture of the dorsal tegument between mesosomal tergites VI and VII. The heamolymph was centrifuged at 3000 rpm for 20 min to avoid coagulation. The supernatant was lyophilized and stored at −20 °C until use.

Experimental animals
Male albino mice weighing 20-25 g were maintained at 25±2°C in the breeding unit of Zoology Department, Suez Canal University under conditions of controlled humidity and on a 12 h-light/dark cycle, with free access to standard laboratory mice chow and water.

Determination of the half lethal dose (LD_{50})
C. cerastes (CC) venom with different known concentrations were injected intraperitoneally into 8 weighted male mice, and then monitoring them to record the mortalility time for each (Meier, J. and Theakston, 1985). Accordingly, LD_{50} of *C. cerastes* venom was estimated to be 1.98 mg/kg.

Experimental design
The present study was conducted on 42 male albino mice allocated in to seven main groups (n= 6), a control group was injected with physiological saline (0.9% NaCl); Four groups were injected intraperitoneally with1/5 LD_{50} (0.396

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mg/kg). The blood and tissue samples were collected 2, 4, 16, 24 h after treatment. The rest two groups of mice received two injections; one of them received [(CC) venom (0.396 mg/kg) + antivenom (polyvalent viper's venom antiserum) (30ml/70kg) or (0.43µl/gm.)]; the anti-venom obtained from Egyptian National Research Center. While, the other group received [(CC) venom (0.396 mg/kg) + (AA) scorpion heamolymph (0.792mg/kg) (2:1)]. The following biochemical parameters were measured in the control and treated groups: malondialdehyde content (MDA) in various body organs (liver, kidney, brain, heart, lung and testes), plasma protein carbonyl contents (PCC), blood and liver glutathione (bGSH and lGSH respectively), plasma nitric oxide (NO), plasma total protein (PTP) and activities of plasma aspartate aminotransferases (AST) as well as alanine aminotransferases (ALT).

Oxidative stress biomarkers

Lipid peroxidation (MDA) content was determined according to the method of Mihara and Uchiyama (1978). Protein carbonyl content was determined according to the method of Levine et al. (1990). Nitric oxide (NO) level was measured in plasma by a spectrophotometric method (Green et al., 1982).

Assessment of non-enzymatic antioxidant

Reduced glutathione (GSH) was estimated in blood and liver according to the method of Beutler et al. (1963).

Liver function tests

PTP was evaluated according to Gornall et al. (1949). ALT and AST were estimated by using commercial kits according to the method of Rightman and Francle (1957).

Statistical analysis

All data were expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA) was carried out followed by *post hoc* Duncan's test; the probability criterion for significance was P ≤ 0.05. Data were analyzed using SPSS® software (Statistical Package for Social Science, version 17, Illinois, and USA) (Dancey and Reidy, 2002).

Results

Oxidative stress biomarkers

Our results revealed that the levels of MDA was significantly increased in all tested organs after 2, 4, 16 and 24h post treatment as compared to the control values. The most significant effect was noticed after 24 h (Table 1) with percentages of increase (+576, +204, +199, +2477, +424 and +255 % in liver, kidney, brain, testis, heart and lung, respectively). PCC concentration recorded significant increase at all-time intervals as compared to the control group (Table 2) and percentage of increase was +325% at 24 h. The data in Table 2 shows highly significant increase in the level of plasma NO at all-time intervals of the treated groups in comparison with the control, the most potent increase detected at 24hours, with a percentage of +1040%. There were significant differences between the treated groups in MDA, PCC and No levels ($P \leq 0.05$).

Antioxidant status

*b*GSH and *l*GSH levels were decreased significantly ($P \leq 0.05$) at all-time intervals as compared to those of control. The most potent effect was recorded at 24 hours with percentage of decrease (-49% and -35% for *b*GSH and *l*GSH, respectively). One-way ANOVA revealed highly significant differences between treated groups as shown in Table 2.

Table 1: Effect of C. cerastes venom on the level of malondialdehyde (MDA) in various organs.

MDA µMol/mg tissue					
	Time post-treatment (Hours)				
Organ	Control	2h	4h	16h	24h
Liver	0.79 ± 0.20 [a]	1.77 ± 0.10	3.09 ± 0.11	4.04 ± 0.38	5.31 ± 0.17*
Kidney	2.48 ± 0.08	4.24 ± 0.13	5.40 ± 0.12	6.57 ± 0.17	7.53 ± 0.10*
Brain	1.87 ± 0.09	1.97 ± 0.13	3.28 ± 0.12	4.20 ± 0.12	5.60 ± 0.37*
Testis	0.20 ± 0.07	2.00 ± 0.10	3.01 ± 0.11	3.87 ± 0.12	5.08 ± 0.26*
Heart	0.69 ± 0.04	0.95 ± 0.09	2.06 ± 0.07	2.88 ± 0.10	3.64 ± 0.13*
Lung	0.61 ± 0.06	0.55 ± 0.08	1.02 ± 0.06	1.53 ± 0.04	2.18 ± 0.15*

aData are expressed as mean +SE (n=6 per group)..

*Significant difference between control and treated groups using one way-ANOVA (p≤ 0.05).

Table 2: Effect of C. cerastes venom on oxidants/ antioxidant status.

Parameter	Time post –treatment (Hours)				
	Control	2h	4h	16h	24h
PPC (nMol/mg)	10.9 ± 1.01 [a]	20.2 ± 2.04	29.2 ± 1.77	39.7 ± 2.66	46.2 ± 4.20*
NO (µM/ml)	1.05 ± 0.18	2.61 ± 0.38	7.86 ± 0.14	8.54 ± 0.18	12.0 ± 0.56*
bGSH (mg/ml)	2183 ± 72.0	1494 ± 34.8	1379 ± 20.3	1225 ± 12.7	1098 ±15.9*
lGSH (mg/mg)	1559 ± 60.0	1242 ± 9.31	1164 ± 7.26	1076 ± 11.6	1011 ± 11.5*

aData are expressed as mean +SE (n=6 per group).

*Significant difference between control and treated groups using one way-ANOVA(p≤ 0.05).

Liver function tests

The data of liver enzymes (AST and ALT) were presented in Fig. 1 and 2. The levels of both ALT and AST in the venom treated mice increased significantly (p≤ 0.05) at all-time intervals as compared to those of control with percentage (+605%, +400%, respectively). The one-way ANOVA revealed highly significant differences between treated groups of all time –intervals (2, 4, 16 and 24 hours). In contrast, the PTP decreased significantly (p≤ 0.05), especially after 24 h (-160%) (Fig. 3).

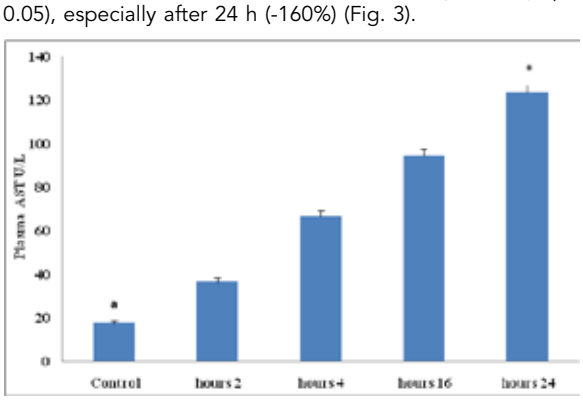

Fig. 1: Effect of C. cerastes venom on plasma aspartate aminotransferase (AST).

[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between control and treated groups using one way-ANOVA (p≤ 0.05).

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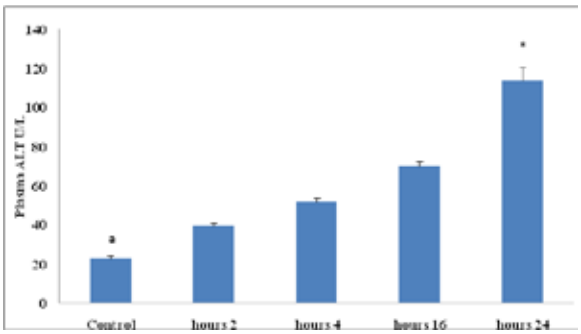

Fig. 2: Effect of C. cerastes venom on plasma alanine aminotransferase (ALT).
[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between control and treated groups using one way-ANOVA (p≤ 0.05).

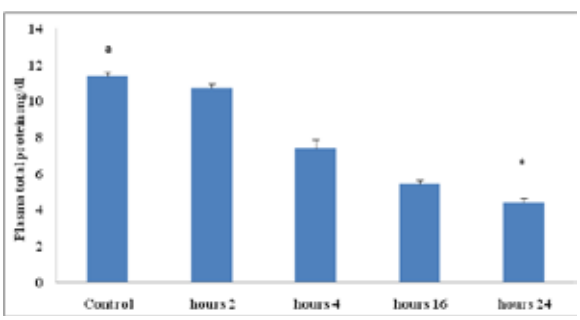

Fig. 3: Effect of C.cerastes venom on plasma total protein.
[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between control and treated groups using one way-ANOVA (p≤ 0.05).

Neutralization results
Oxidative stress biomarkers:
Table (3) indicating the neutralization of MDA in different organs. There was significant depletion with anti-venom and AA scorpion heamolymph compared with venom treated group (p≤ 0.05). The percentages were -78, -65, -67, -55, -67, -80% in liver, kidney, brain, testis, heart and lung, respectively) for anti-venom and -69, -60, -62, -42, -56, -82% in liver, kidney, brain, testis, heart and lung, respectively for heamolymph. Effects on PPC and NO were showed in the Table 2, PPC and NO levels were significantly decreased in anti-venom and heamolymph treated groups compared with the venom group with percentages of -47 and -54% in anti-venom and heamolymph groups, respectively for PPC and -34.7 and -36.7% in anti-venom and heamolymph groups, respectively for NO. Results revealed no significant differences between anti-venom and heamolymph effects on all parameters.

Antioxidant status
Data revealed significant increase in levels of *b*GSH and *l*GSH in both anti-venom and heamolymph groups compared to venom treated group, with no significant differences between their neutralizing effects. Percentages of changes were +12.8 and +12% for bGSH and +17.8 and +16.3% for lGSH in anti-venom and heamolymph groups, respectively (Table 4).

Table 3: Comparison between neutralizing effects of antivenom and AA scorpion heamolymph on MDA in various organs

MDA µMol/mg tissue			
	24 hours post-treatment		
Organs	Venom	Antivenom	Heamolymph
Liver	5.31 ± 0.17 [a]	1.16 ± 0.23	1.60 ± 0.38*
Kidney	7.53 ± 0.10	2.64 ± 0.27	3.03 ± 0.31
Brain	5.60 ± 0.37	1.84 ± 0.24	2.10 ± 0.07
Testis	5.08 ± 0.26	2.29 ± 0.26	2.90 ± 0.51
Heart	3.64 ± 0.13	1.19 ± 0.18	1.59 ± 0.21
Lung	2.18 ± 0.15	0.97 ± 0.09	0.90 ± 0.54

[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between treated groups (venom, antivenom & haemolymph) using one way-ANOVA (p≤ 0.05).

Table 4: Comparison between neutralizing efficacy of antivenom and AA scorpion heamolymph on oxidants/ antioxidant status.

Parameters	24 hours post-treatment		
	Venom	Antivenom	Heamolymph
PPC (nMol/mg)	46.2 ± 4.20 [a]	24.2 ± 4.04	21.2 ± 3.40
NO (µM/ml)	12.0 ± 0.56	7.82 ± 1.45	7.58 ± 0.76
bGSH (mg/ml)	1098 ±15.9	1238 ± 29.3	1231 ± 25.3
lGSH (mg/mg)	1011 ± 11.5	1191 ± 19.9	1177 ± 18.8

[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between treated groups (venom, antivenom & haemolymph) using one way-ANOVA (p≤ 0.05).

Liver function tests
The activity of both AST and ALT has been significantly decreased in the treated groups of antivenom (-39.5 and -55.8 %, respectively) and haemolymph (.2 and -54.2%, respectively) compared with levels in the venom group (Fig. 4,5). Reversely, PTP showed a very significant increase (+152 and +125% in anti-venom and heamolymph groups, respectively) compared to venom treated group (Fig.6). There was a significant difference between anti-venom and heamolymph groups only in the case of PTP.

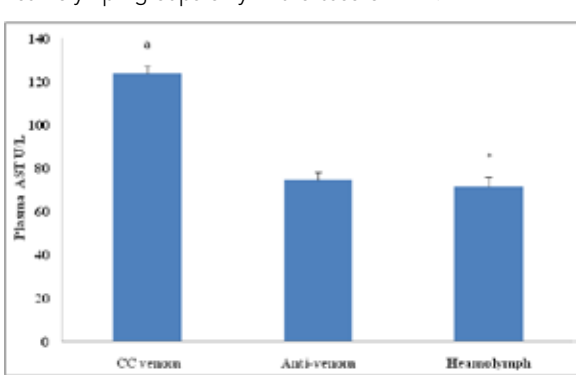

Fig. 4: Comparison between neutralizing effects of antivenomand AA scorpion heamolymph on plasma AST.
[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between treated groups (venom, antivenom & haemolymph) using one way-ANOVA (p≤ 0.05).

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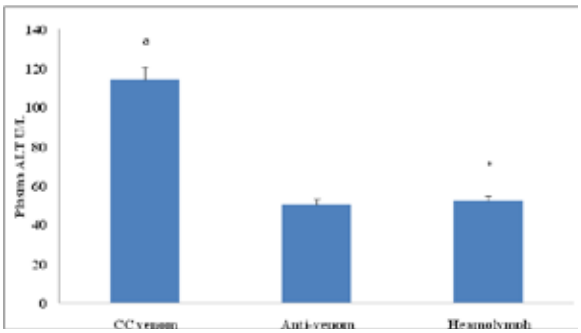

Fig. 5: Comparison between neutralizing effects of antivenom and AA scorpion heamolymph on plasma ALT.

Data are expressed as mean +SE (n=6 per group). *Significant difference between treated groups (venom, antivenom & haemolymph) using one way-ANOVA (p≤ 0.05).

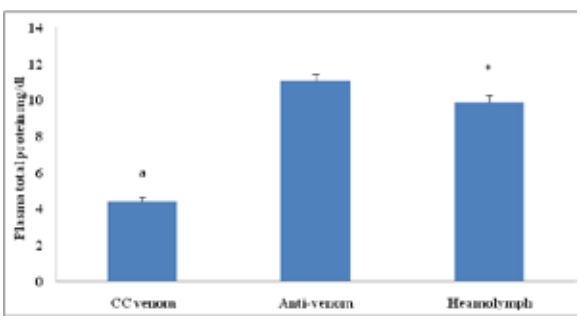

Fig. 6: Comparison between neutralizing effects of antivenom and AA scorpion heamolymph on plasma total protein.

[a]Data are expressed as mean +SE (n=6 per group). *Significant difference between treated groups (venom, antivenom & haemolymph) using one way-ANOVA (p≤ 0.05).

Discussion

Viper venom is a complex mixture of enzymatic and toxic proteins**.** *C.cerastes* contains different enzymes displaying proteolytic activity and causes many toxicities (Aird, 2002, Anai et al., 2002 and Warrell, 2004). The lethal effects of snake venom are largely attributed to its active ingredient phospholipase A2 (PLA2). Phospholipid hydrolysis by PLA2 enzyme releases arachidonic acid whose metabolism results in the formation of potentially toxic reactive oxygen species (ROS) and lipid peroxides (Adibhatla et al., 2003; Neuzil et al., 1998). El Asmar et al. (1979) reported that the increase in polyunsaturated fatty acids following envenomation may lead to an increase in the rate of lipid peroxidation, which might be responsible for tissue damage. Oxidative stress mediated by an imbalance between ROS production, and detoxification may also disturb cellular physiology and is incriminated in the pathophysiology of many diseases (Funasaka et al. 2012). The results of this study clearly demonstrated that a single injection of CC venom at a dose of 0.396 mg/kg body weight caused a significant and persistent increase in MDA content in all studied organs. A significant increase in lipid peroxidation product was observed within 2 hrs of venom injection and the most potent increase detected after 24 hrs. Carbonyl content of proteins is an index for protein damage, particularly those associated with oxidative stress (Levine et al., 1990). In the present study, we have reported for the first time the oxidative protein damage after CC envenomation. Moreover, a highly significant increase was observed in PCC and NO in CC treated-animals. So, the increase in PCC might be in part due to the increase in the NO concentration. This result is consistent with the fact that venoms are known to trigger the release and activation of pro-inflammatory cytokines and other mediators, such as nitric oxide (Petricevich, 2004). NO is readily reacts with superoxide to form peroxynitrite, a potent oxidant and nitrating agent capable of attacking and modifying proteins, as well as depleting antioxidant defenses (McCord, 2000). These results were in accordance with (De-Castro et al., 2004). Glutathione plays a fundamental role in the redox system balance and involved in molecular protective mechanisms (Bilska et al., 2007). Our results revealed a significant depletion in the levels of GSH in both blood and liver in the venom treated animals which was probably necessary for host defense to encounter deleterious effects of ROS. The enhanced MDA content may also be due to marked depletion of GSH content of liver, which acts as one of the guarding factors against oxidative stress (Levine, 1990). Our biochemical results revealed a highly significant increase in ALT and AST. The most increase was detected after 24hrs which reflected liver damage. These results are matching with Al-Jammaz 2001 and Al-Sadoon et al., 2013. Hepatocellular damage occurs when the membrane of hepatocytes loses integrity, releasing aminotransferases to the plasma. A possible cause for alterations in membrane permeability is oxidative stress (Babcock et al., 1981). On the other hand, our findings detected sever decrease in plasma total protein which is an indicator for hepatic function.

Anti-venoms are commonly used to treat the snakebites. Degradation of local tissue damage is a continuous process which is prolonged even after the administration of antivenom (Rucavado et al., 2004). Disadvantages of using classical antivenom are high cost, hypersensitivity, non-availability and lack of protection against local effects increasing the urge to find a new generation of therapeutic agents. A number of studies have reported that snake sera and scorpions heamolymph have cross-species toxin inhibitory effects (Smith et al., 2000). From this point of view, we tried to examine the neutralizing efficacy of the Egyptian *A. australis* scorpion heamolymph against CC venom and compare it with anti-venom efficacy. Treatment by antivenom or heamolymph after the venom injection induced a significant depletion in oxidative stress biomarkers; MDA, PPC and NO. On the other hand, both blood and liver GSH were increased significantly referring the venom neutralization offered by either antivenom anti-venom or heamolymph. Our data revealed that the levels of both AST and ALT detected a high significant depletion with antivenom and heamolymph. In contrast, PTP revealed a very significant increase for the anti-venom and heamolymph treated groups compared with the venom group. There was no difference between the neutralizing effects of the anti-venom and AA scorpion heamolymph. Thus, we can conlude that there may exist antitoxic proteins in the blood of this scorpion that can neutralize the toxicity of *C. Cerastes* venom.

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