University of Benghazi
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جامعة بنغازي
كلية التربية – المرج
ISSN 2518-5845

Global Libyan Journal

المجلة الليبية العالمية

العدد الخمسون / يناير / 2021

Effect of *Naja haje* (Egyptian cobra) crude venom- induced oxidative stress on the kidney of Wister albino rats

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Abstract

In this study, oxidative stress inductions as well as nephrotoxicity of the Egyptian cobra crude venom at a doses of 0.025mg/kg (i.m.) of rats after 3, 6 and 9 h from envenomation. 90 rats divided into 3 groups, control group injected with 200 µl saline solution and group (2) injected with LD50 of cobra crude venom group (3) injected with1\2 LD50 of cobra crude venom . Quantitative evaluation of functional alterations in the kidneys were performed by biochemical analyses. Values of urea and creatinine significantly increased (P≤0.05) LD50 and ½LD50 at 3 h,6 h and 9 h. Oxidative stress biomarkers were assayed in serum levels of MDA was significantly at 3 h and nitrite 9 h while the levels of GSH, SOD and CAT were significantly decreased, especially after 9 h of envenomation. The levels of MDA and NO in serum were markedly increased especially at 3h and 9 h respectively, whereas The levels of serum GSH, SOD and CAT were decreased after 9 h respectively. These results suggest that, such effects which are so drastic may reflect probabilities of nephrotoxicity due to cobra envenomation with the involvement of oxidative stress as a potential mechanism.

الملخص :

تهدف الدراسة لتقييم الإجهاد التاكسدي كاليه لإحداث السمية الكلويه الناجمة عن سم الكوبرا المصرية المجفف.من خلال الحقن العضلي لجرعتين من السم (الجرعة النصف قاتله 2\1& الجرعة نصف القاتلة).90 جرذ استخدم في هذه الدراسة حقنت بالسم علي فترات زمنيه مختلفة (6,3 و9 ساعات).قسمت لثلاث مجموعات الأولي استخدمت كمجموعه ضابطه,ا لثانيه حقنت الجرعة النصف قاتله والثالثة عوملت الجرعة الأخرى. قتلت 10 جرذان بعد 6,3و9 ساعات من الحقن . وتم إجراء التحاليل لقياس وظائف الكلي في السيرم, وسجلت اليوريا والكرياتينيين ارتفاع معنوي عند الجرعتين وللفترات الزمنية الثلاث وكانت عالية المعنوية عند 9 ساعات مقارنة مع المجموعة الضابطة. بالإضافة لذلك كشفت النتائج ان لسم الكوبرا نشاط مؤكسد قوي حيث لوحظ زياده معنوية في مستويات المولوداي الدهيدوالنيتريت ,وانخفاضا معنويا في مستوى مضادات الاكسده (الجلوتاثيون, فوق اكسيد الديسموتيزو الكاتليز). الا انه المولوداي الدهيد والنيتريت اظهر ارتفاع معنوي عالي بعد 3و9 ساعات علي التوالي.في حين سجلت مستويات مضادات الأكسدة انخفاضا معنويا عاليا بعد9 ساعات. تشير هذه النتائج إلى أن مثل هذه التأثيرات شديدة الخطورة قد تعكس احتمالات السمية الكلوية لسم الكوبرا المصريه الناتج عن الاجهاد التاكسدي كالية محتمله ..

الكلمات المفتاحيه : الإجهاد التاكسدي,الناجا هاجا, سم, السمية الكلوية, الجرعه نصف القاتله.

Introduction

Snakebite envenomation is known to man since antiquity and many references to snakebite are found in the oldest medical reports. There are more than 2.5 million venomous snake bites annually, with greater than 125000 deaths, the risk is highest in the tropics and West Africa, predominantly among rural population [25,33,45,56]. Most cobras are generally diurnal and they live close to human dwellings, in agricultural fields and water courses where prey are found, Postsynaptic toxins are the main lethal principle in cobra venom the clinical manifestations: severe neurotoxicity with mild local reactions the muscle paralysis is typical of the syndrome and repetitive nerve stimulation shows a decrement abolished by edrophonium[13,43,54]. the onset of systemic effect of envenomation. Ptosis, frothy saliva, slurred speech, respiratory failure, and skeletal muscle paralysis are outcomes of the neuromuscular effect of venom proteins within 8-19 hours of cobra bite [9,44] Cobra envenoming is known to induce multiple-organ failure, leading to death in case of severe envenoming [11,12]. Histological, histochemical and biochemical fluctuations triggered by the venom of Egyptian cobra (*Naja haje*) have already been evaluated on rodent animal [19, 29]. In severe cases, tissue damage reaches many organs, such as the brain, lung, kidney, heart, and liver [31, 36]. Nephropathy induced by cobra factor was mentioned by [37]. and whereas Acute kidney injury due to snake venom is a neglected condition around the world that needs to be studied and analyzed to know the mechanism of its occurrence, one of the importants oxidative stress.[8,21] The persistence of oxidative stress damages vital organs, such as the liver and kidneys, resulting in multiple organ failure, Nevertheless, the oxidative stress induced by the venom of *N. haje* was not sufficiently covered in the available literature [6,18]. Thus, this study was conducted to examine the possible acute toxicity effect of LD_{50} and $_{1\backslash 2}LD_{50}$ of the *Naja haje* crude venom on kidney of rats at intervals of time, unveiling the oxidative stress mechanism induced by venom.

Materials and Methods

Materials

Animals

Ninety adult male rats body weight of (200 ± 20g) were used in the experiment. The rats were maintained under control room temperature of 22 ± 3C0 with 12 hours light/dark cycles and

the humidity level of 50- 60%, The rats were fed with standard pellet diet and water ad libitum was provided throughout the study. Composition of standard pellet diet which was fed to the rats contained corn, pulp of soybean, seeds of sunflower, molasses, meat-bone meal, marble dust, vitamins and minerals. The composition of nutrients was crude protein (23%), crude fat (3%), crude fiber (7%), acid insoluble ash (8%), calcium (1%-2.5%), phosphorus (0.9%), sodium (0.5%-1%) and moisture (12%).

Venom

Lyophilized *Naja haja* venom was obtained from Egypt (Center of serum and vaccine in Alexandria). Lyophilized venom were dissolved in phosphate buffered saline (PBS), pH7.4 Contents of one liter (8 g of NaCl, 0.2 g , KCl and 0.24 g of KH2PO4) was obtained from Sigma.

Methods

Determination of LD_{50} dose

LD**50** of crude venom determine as describe by [39]. LD50 of the venom were determined by (i.m) injection of different concentrations of venom in 0.1 ml of phosphate buffered saline (PBS) After the lethal dose is determined, each group is injected with a different concentration, the space the dosage levels so that they are in a geometric progression . Mortality rates after injection were (0,0,2,4). The general formula for the calculation of (m),the estimate of LD50 may be reduce to:

m= The median lethal does (LD50) . n= The Number dosed per level.

D=The log of the lowest of the four dosage levels used. d= The logarithm of the constant ratio between dosage levels. f = Mortality rate in groups table [39]. The LD50 toxicity values of collected the Egyptian cobra *Naja haja* were assessed by injected (i.m) rats and Calculated by:

n= 4 , d= 2 ,D= 63.50 , f = 1

$$log\ m \cong log\ 63.50 + 2\ (1 + 1)$$

LD50 = 0.25mg/kg

The LD**50** of crude venom of the Egyptian cobra in rats was found to be 0.25mg/kg.

Experimental design

Animals were divided into three experimental groups:

Control group: Rats intramuscularly (i.m.) injected only with 0.l ml phosphate buffered saline (PBS) without venom, and was killed after 9 hours from the injection.

LD50-Envenome group: Rats i.m single does injected with 0.1ml phosphate buffered saline(PBS) containing LD50 (0.25mg cobra venom / kg body weight of the rat). The rats was subdivided to three subgroups (ten rates each) was killed after 3, 6 and 9 hours from envenoming respectively. ½LD50-Envenome group: Rats i.m single does injected with 0.1ml phosphate buffered saline (PBS).Containing ½LD50 (0.125mg cobra venom /kg body weight of the rat). This group subdivided to three subgroups (ten rats each) was killed after 3 , 6 and 9 hours from envenoming respectively.

Methods were used for Biochemical

Blood samples was collected in sterile label heparinize 5ml test tubes through the neck blood vessels and allow to stand for 2 hours at room temperature then centrifuge at 2000 rpm for 10min. The clear supernatant used for the various biochemical determinations. Estimation of kidney functions and other Biochemical colorimeter measure analysis using the method of kits.

kidney functions test:

The urea and creatinine at Serum, Colorimeter measure analysis using the method of kits provide from (BIODIAGNOSTIC).

Nitrosative and Oxidative stress markers:

Nitrite/nitrate (nitric oxide; NO) and lipid peroxidation (MDA) levels were determined by calorimetrically in the serum according to the methods of [13, 30 , 14]. Colorimeter measure analysis using the method of kits provide from (BIODIAGNOSTIC).

Antioxidant Biomarkers:

The activity of glutathione (GSH) and Superoxide dismutase (SOD) and catalase (CAT) in the serum was determined by the method of [29]. and methodology of [4]. Colorimeter measure analysis using the method of kits provide from (BIODIAGNOSTIC).

Statistical analysis

The data were analysis's used Minitab version 16. Mean ± SE is given for quantitative variables. One way ANOVA was used to compare the groups and Tukey post hoc test was used for detail analysis. (version 2.0.288).

Results

Biochemical results

In the present work, showed that Rats envenomated with the Egyptian cobra crude venom at injected (i.m) for the 3, 6 and 9 hours, induced significant differences in serum levels of urea and creatinine.

The level of Urea in Serum

Envenomation by different doses of injected (i.m) (LD50 and 1/2LD50) resulted in a significant elevation (P≤0.05) in urea and creatinine levels as compared to its control group table (1&2). The injection of crude venom LD50 group2 and 1/2LD50 group3 led to significant increases in serum urea compared with control after 3, 6 and 9 hours versus (22±0.34)These increases were (48±0.30),(52±0.12) and (59±0.09) in table (1).

After injection and the increases of group3 were (42±0.11),(46±0.32) and (50±1.21) versus (22±0.34). after 3,6 and 9 hours respectively, post-injection as compared with control. Serum levels of urea with (LD50) and (1/2LD50) at 9 h were considered highly significant compared with the control group at (P≤ 0.01), (59±0.09) &(50±1.21) versus (22±0.34) respectively.

Table (1) levels of serum (urea) of Rats which induced the Egyptian cobra crude venom (LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	urea(mg/dl)		
	3hr	6hr	9hr
Control(group1)	22±0.34	22±0.34	22±0.34
LD50(group2)	48±0.30*	52±0.12*	59±0.09**
1\2LD50(group3)	42±0.11*	46±0.32*	50±1.21**

Values are means ± S.E. (*) significant against control group at P ≤ 0.05 and (**)highly significant at P ≤ 0. 01.

The level of Creatinine in Serum

The injection of crude venom LD_{50} group2 and $_{1/2}LD_{50}$ group3 led to increases in serum creatinine levels at the 3,6 and 9 hours after injection compared with the control group ($P \leq 0.05$). Table (2)

creatinine levels at 9 h with (LD_{50} and$_{1/2}LD_{50}$) venom were highly significant $P \leq 0.01$. (1.95±0.10) and (1.80±0.08) as compared with control (0.56±0.09) respectively. The injection of crude venom LD_{50} group2 and $_{1/2}LD_{50}$ group3 led to significant ($P \leq 0.05$) increases in serum (1.38±0.04),(1.93±0.03) and (1.17±0.02),(1.78±0.031) at 3 and 6 hours, after injection as compared with the control group (0.56±0.09) respectively.

Table (2) levels of serum creatinine of Rats which induced the Egyptian cobra crude venom(LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	Creatinine (mg/dl)		
	3hr	6hr	9hr
Control(group1)	0.56±0.09	0.56±0.09	0.56±0.09
LD50(group2)	1.38±0.04*	1.93±0.03*	1.95±0.10**
1\2LD50(group3)	1.17±0.02*	1.78±0.031*	1.80±0.08**

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and (**) highly significant at $P \leq 0.01$.

The level of malondialdehyd (MDA) in serum

A significant ($P \leq 0.05$) increases was recorded in the MDA level on the 3h,6h and 9h due to the venom injection at a doses levels LD_{50} and $_{1/2}LD_{50}$ compared with the control group table (3). The statistically of these increases were: (148.32±0.52),(147.15±0.32) , (146±1.92) and. (139.42±0.61),(136±1.03) and (134±1.34) as compared with control group (117.20±1.03) after 3, 6 and 9 hours of injection, respectively. A highly significant ($P \leq 0.01$) increase at LD_{50} and $_{1/2}LD_{50}$ doses was noticed on the 3 h as compared to the control group (148.32±0.52) & (139.42±0.61) versus control group (117.20±1.03).

Table (3) levels of serum (MDA) of Rats which induced the Egyptian cobra crude venom (LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	MDA(ng/l)		
	3hr	6hr	9hr
Control(group1)	117.20±1.03	117.20±1.03	117.20±1.03
LD50(group2)	148.32±0.52**	147.15±0.32*	146±1.92*
1\2LD50(group3)	139.42±0.61**	136±1.03*	134±1.34*

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and (**) highly significant at $P \leq 0.01$.

The level of nitrite/nitrate (NO) in serum

The NO level showed a significant ($P \leq 0.05$) elevation resulting from the envenomation at all the investigated doses and time intervals in the serum of rats compared with the control group table (4).These increases of group2 and group3 were (22.9±1.08),(25.1±0.05) and (29.41±0.6) compared to the control group (13±0.04) and (20.3±0.070),(23.1±0.12) and (26±1.05) compared with control (13±0.04). after 3, 6 and 9 hours of injection, respectively. But the level of (NO) of envenomated Rats at (LD_{50} and$_{1/2}LD_{50}$) doses was highly significant ($P \leq 0.01$) increase in serum at the 9 hour (29.41±0.6) & (26±1.05) compared to control (13±0.04).

Table (4) levels of serum (NO) of Rats which induced the Egyptian cobra crude venom (LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	NO (µmol/ml)		
	3hr	6hr	9hr
Control(group1)	13±0.04	13±0.04	13±0.04
LD50(group2)	22.9±1.08*	25.1±0.05*	29.41±0.6 **
1\2LD50(group3)	20.3±0.070*	23.1±0.12*	26±1.05**

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and (**) highly significant at $P \leq 0.01$.

The levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT).

The result in table (5,6&7) showed that levels of GSH,SOD and CAT in the serum of the control and injected (i.m) rats. Envenomation by different doses of injected (i.m), (LD_{50} and $_{1/2}LD_{50}$) resulted in a significant reduction in the GSH,SOD and CAT levels at all the time intervals as compared to control group.

level of glutathione (GSH) in serum

The venom effects on serum GSH levels led to decrease after 3 h,6 h and 9 hours post-injection in LD_{50} group2 and $_{1/2}LD_{50}$ group3.Table (5). The statistically of these decreases were ($P \leq 0.05$). (13±0.08),(12.21±0.06); (11.55±1.05) ;(15.18±0.14), (14.78±0.32) and (13.32±0.16) compared with control group (30.8±0.42) respectively. But a highly significant($P \leq 0.01$) for each of the doses at the 9 hour, these decreases were (11.55±1.05) and (13.32±0.16) compared with control group (30.8±0.42) respectively.

Table (5) levels of serum (GSH) of Rats which induced the Egyptian cobra crude venom (LD_{50}&$_{½}LD_{50}$) of (i.m) injection at different times intervals.

Parameters / Groups	GSH(nmol/ l)		
	3hr	6hr	9hr
Control(group1)	30.8±0.42	30.8±0.42	30.8±0.42
LD50(group2)	13 ± 0.08*	12.21±0.06*	11.55±1.05**
1\2LD50(group3)	15.18±0.14*	14.78±0.32*	13.32±0.16**

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and(**) highly significant at $P \leq 0.01$.

The level of superoxide dismutase (SOD) in serum

Both groups were statically significant ($P \leq 0.05$) decrease at 3 and 6 hr. (2.36±0.05),(2.44±0.11), (3.35±0.09) and (3.35±0.09) in level of serum SOD as compared with control group (5.90±0.09) respectively. While these is highly significant($P \leq 0.01$) decreases at the 9hour. (2.05±0.20) & (2.83±0.41) compared with control group (5.90±0.09) respectively.

Table (6) levels of serum (SOD) of Rats which induced the Egyptian cobra crude venom (LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	SOD(IU/L)		
	3hr	6hr	9hr
Control(group1)	5.90±0.09	5.90±0.09	5.90±0.09
LD50(group2)	2.36±0.05*	2.44±0.11*	2.05±0.20**
1\2LD50(group3)	3.35±0.09*	3.35±0.09*	2.83±0.41**

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and (**) highly significant at $P \leq 0.01$

The level of catalase (CAT) in serum

Rats treated with (LD_{50}&$_{1\backslash2}LD_{50}$) of venom showed significant ($P \leq 0.05$) reduced in CAT levels (40.17±1.81),(38.20±1.32), (49.35±0.17) and (42.72±0.12) at 3and 6hrs respectively compared with control group (64.15±1.36) table (7). However , both groups showed highly significant ($P \leq 0.01$) reduced in CAT levels at 9 hrs (32.29±1.72) and (39±1.82) as compared with control group (64.15±1.36) respectively.

Table (7) levels of serum (CAT) of Rats which induced the Egyptian cobra crude venom (LD_{50}&½LD_{50}) of (i.m) injection at different times intervals.

Parameters / Groups	CAT (IU/L)		
	3hr	6hr	9hr
Control(group1)	64.15±1.36	64.15±1.36	64.15±1.36
LD50(group2)	40.17±1.81 *	38.20±1.32 *	32.29±1.72**
1\2LD50(group3)	49.35±0.17 *	42.72±0.12 *	39±1.82**

Values are means ± S.E. (*) significant against control group at $P \leq 0.05$ and (**) highly significant at $P \leq 0.01$.

Discussion

In the present investigation, we explored the systemic physio pathological changes induced by the Egyptian cobra venom in kidney as a vital organ. Urea and creatinine are nitrogenous waste products that are eliminated by the kidneys, whereas excretion is suppressed in renal insufficiency [27,35]. Our results revealed that, The high levels of creatinine and urea indicate an impairment of renal function by significant ($P \leq 0.05$) increase in serum creatinine and urea levels in two different doses of crude venom injected (i.m) (LD50 & 1\2 LD50) after time intervals. This was in agreement with the findings of [42 , 57 , 46]. The significant increase in serum urea and creatinine levels in the present study may be due to the nephrotoxic effect of Egyptian Cobra (Naja haje). This was in agreement with the findings of [42 , 57]. They mentioned that serious renal complications in case of Naja haje and Cerastes cerastes envenomation lead to impairment of the excretory function of the kidney. Significant elevation in serum urea recorded in this work may be also attributed to an increase of nitrogen retention and / or due to corrupted renal function [2] Moreover, there was a reduction in GSH,SOD and CAT levels after time.

intervals, regarding MDA and NO, the increase in its levels was significant at 3, 6 and 9 hours with (LD50 & 1\2 LD50). The present show that, the nephrotoxicity effect of *Naja haje* venom crude was intensified with increasing dose and intervals, except the MDA were increased after injection 3 hr for both doses. The free radicals, apart from being involved in damaging cellular components, do play a significant role in venom induced toxicity [34,35]. Venomous snakes have enzymes that can directly cause cellular injury [17] There are no available data regarding the involvement of oxidative stress induced by *Naja haje* venom. In order to evaluate the ability of *Naja haje* venom crude to produce oxidative stress, we choose to monitor one of the earliest responses of oxidative stress, which is the increase of stress markers in serum, including antioxidant enzymes, nitric oxide and lipid peroxidation. The present study showed that, the envenomation of rat with *N. haje* venom crude after 3, 6 and 9 hours a highly significant increase in lipid peroxidation (MDA) and nitrite/nitrate (NO) levels as compared with control group. However, *N. haje* venom crude venom injection induced a highly significant reduction in levels of GSH, SOD and CAT activity after the three selected time intervals and were only highly significant in 9 hours. Oxidative stress maybe a result of

excessive reactive oxygen species generation or failure of the cellular antioxidant system. Snakes venom induced an elevation of oxidative stress indicators as nitric oxide and lipid peroxidation [4,5]. Glutathione is widely distributed tri peptide and found mainly in the cell cytosol [33]. Glutathione is the cell's natural antioxidant, which destroys free radicals formed in cells. This plays a crucial role in the detoxification process. Our results supported by the previous. interpretation of the consequences of the GSH deficiency which causes oxidant damage and greater lipid peroxidation which in turn leading to cell damage [53, 47, 10]. The venom-evoked enhanced MDA and free-radical generation observed in this study may elucidate the deleterious organ defects and multiple organ disorders observed following snake envenomation. Snakebites are most often accompanied by signs of inflammation and local tissue damage. Neutrophils and macrophages are stimulated to produce superoxide radical anion which belongs to a group of ROS. This reacts with cellular lipids leading to the formation of different peroxides and subsequently leading to tissue necrosis. As the origin of oxidative stress is the mitochondrial respiratory electron transport chain, it is possible, that mitochondrial death potentiates venom-induced cellular damage [24,52].

The induction of the enzymatic antioxidant defenses after the exposure to *N. haje* venom could be considered as an adaptive response that is, a compensatory mechanism that enables cells to overcome the damage caused. To further demonstrate the implication of oxidative stress in venom induced toxicity. L-amino acid oxidases (LAAOs) are flavor proteins that are able to catalyze the oxidative deamination of L -amino acids to produce the corresponding α-keto acids along with the concomitant release of hydrogen peroxide (H2O2) and ammonia. Although they occur in many different organisms from invertebrates to vertebrates, their functions in vivo are uncertain. LAAO is widely distributed in venomous snakes including the viper and elapids and is thought to contribute to their toxicity, possibly through H2O2 formed as a result of reoxidation of the transiently reduced FAD cofactor by molecular oxygen [30,15]. The enzyme is the major component of snake venoms, and in some species this enzyme alone constitutes approximately 30% of the total protein content [17 , 15]. Furthermore, venom LAAO has been shown to induce cell death in several mammalian cell lines [7,16,39,48]. The effect was attributed to the formation of localized high concentrations of H2O2, a known reactive oxygen species (ROS). It is interesting to

note that the LAAO induced apoptosis has been reported to be different from that caused by exogenous H2O2, suggesting that the mode of delivery of H2O2 is an important factor[28,49]. In addition, snake venom LAAOs appear to be cytotoxic against many organisms [39, 51]. suggested that cells submitted to oxidative stress induced by LAAO generated H2O2 that could activate heat shock proteins and initiate cell membrane disorganization, DNA fragmentation, apoptosis and therefore cell death [50]. Internal and external mitochondrial membrane permeability (MMP) changes led to disappearance of MMP and release of cytochrome c and other pro-apoptotic factors into the cytosol. The release of pro-apoptotic factors in the cytoplasm may initiate apoptosis cascade reaction, which includes activation of caspase-3 and other substances that trigger proteolytic enzymes and break DNA into fragments [1,49].

In this study we discussed one of the mechanisms that may be the cause of kidney poisoning caused by the poison of the Egyptian cobra and there are still many mechanisms that can be studied that may be the cause of this process. As well as studying the components of the toxin separately, one of them may be the main responsible for toxic events.

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