Pakistan J. Zool., vol. 45(5), pp. 1359-1366, 2013

Toxic Effects of Crude Venom of a Desert Cobra, *Walterinnesia aegyptia,* on Liver, Abdominal Muscles and Brain of Male Albino Rats

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Abstract.- The toxic effect of an acute dose of *Walterinnesia aegyptia* crude venom was studied in male albino rats. Liver enzymes,alaninetransaminase (ALT), aspartate transaminase (AST) and gamma glutamyltransferase(γ-GT), total protein concentration and Alkaline phosphatase(ALP) enzyme activity in the liver, abdominal muscles and cerebrum brain were measured at timed intervals of 1, 3, 6, 12, 24, 72 h and 7 days post envenomation. The histological changes in the liver sections were simultaneously investigated. These parameters were found to be fluctuated with time, with a tendency to regain to normal control levels within the first 6 h. Histological changes induced by treatment with LD_{50} of *W. aegyptia* crude venom in liver 3 to 6 hours post envenomation showed inflammatory cellular infiltrations(ICI) around the hepatic vein, dilated blood sinusoids (S), hepatocyticvacuolations (HV) and prominent van kuffer cells. The 12 to 24 h period seems to be crucial for the process of physiological recovery. Histological changes induced by treatment with LD_{50} of *W. aegyptia* crude venom in liver 12, 24, 72 hrs to 7 days post envenomation showed hepatocytic-vacuolations, inflammatory cellular infiltration and dilated sinusoids. Under higher magnification, marginal chromatin (mc) patterns appear in some hepatocytes and clumped chromatin (cm) in others. With this same group, liver sections taken at 72 h and at 7 days showed increased inflammation and vacuolation as evidenced by an increase in inflammatory cells, some pyknotic cells (Pn), widened sinusoids and numerous van Kupffer cells. Fatty change or Steatosis(St) represents the intracytoplasmic accumulation of triglycerides (neutral fats) of parenchymal organs. Physiological adaptation and recovery from an LD_{50} venom dose seems to be achieved after one week, leaving the animal alive with several lesions especially in the liver (such as pyknotic nuclei, steatosis and clumped chromatin and disturbed physiological profile).

Keywords: Biochemical parameters, Crude venom, LD_{50}, Liver enzymes, Liver histological changes, *Walterinnesia aegyptia.*

INTRODUCTION

Annually, there are more than 2.5 million cases of snake bite and mostly in rural tropical areas. Of these about 100,000 are fatal (White, 2005). Disturbances of haemostasis as caused by members of several genera from all four snake families are among the most severe effects following snake bite (Ducancel and Goyffon, 2008). *Walterinnesia aegyptia* a monotypic, elapid snake found in Africa and as well as in the sandy areas of Kuwait, Syria, Saudi Arabia, Lebanon, Jordan, Iran, Iraq, and Egypt (Russell, 1991). Desert black snake *W. aegyptia* inhabits arid hilly terrain, gravel plains and scrubland. They are nocturnal and fossorial. Prey consists of lizards. Only prolonged disturbance or restraint would result in a bit. This snake has been responsible for human fatality and bites should be treated promptly (Egan, 2002). Envenomation by *W. aegyptia* is known to cause rapid deaths and paralysis (Tsai, 2008). The venom of *W. aegyptia* was found to contain various enzymes including phospholipases A2 (PLAs) (Simon and Bdolah, 1980), L-amino acid oxidase, and proteolytic enzymes (Gitter and de Vries, 1968). Three-finger toxins, non-enzymatic proteins of 60–74 amino acid residues, were thought to be present only in elapid venoms, but their presence was recently demonstrated in colubrid and viperid venoms as well (Doley *et al.,* 2008). Despite structural similarity, they differ widely in their activities, being mostly cardio- and neurotoxic but some of them also act on the haemostatic system (Kini,

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2002; Kini and Doley, 2010).

Venom from the *W.aegyptia* and *Pseudo cerastes persicus fieldi* have been found to have the highest hemolytic activity of the elapids and vipers and also possessed the highest phospholipase A2 activity (El-Hakim *et al*., 2008). *W. aegyptia* was reported to induce elevation in serum total proteins, glucose and to alter enzyme activities (Al-Jammaz *et al.,* 1992). Also disorder was seen in liver enzyme activities, total protein and glycogen content. Dramatic disturbance of kidney glycogen, total protein content and enzyme activities in the kidneys of envenomated animals was reported (Al-Jammaz *et al.,* 1994).There was observed reduction in serum total albumin, uric acid, cholesterol and phosphorus along with calcium levels and disturbances in serum electrolyte levels (Al-Jammaz, 2001).

Metabolic activity of cultured human fibroblasts was also affected (Al-Saleh, 1996). *E. coloratus* venom (envenomated by direct biting on sheep muzzles) has been reported to elevate glucose, AST, ALT, triglyceride and total bilirubin while reduce cholesterol. The histological alterations were mainly pyknosis, karyorrhexis, cytoplasmic vacuolation, necrosis, fatty changes and hepatocyte atrophy. Sinusoidal dilatation, Kupffer cell activation, amyloidosis, portal vein thrombosis, partial glycogen depletion and hepatic architecture distortion were also detected in adult male *Ovisorientalis* sheep (Jarrar, 2011). This study aims to determine the biological effects of an acute LD_{50} dose of *W. aegyptia* crude venom at time intervals of 1, 3, 6, 12, 24 and 72 h, as well as a week after envenomation of male rats. This will be achieved through monitoring of changes in certain tissue biochemical parameters and histological changes in the liver.

MATERIALS AND METHODS

Venom collection and preparation

The venom was obtained from the desert cobra, *W. aegyptia.* Specimens were kept in the serpentarium at the Zoology Department, College of Science, King Saud University. These snakes were collected from the central region of Saudi Arabia by a professional hunter. Ten adult snakes were kept in large tanks, heated daily from a 100 watt lamp for a period of 9 h and had access to water *ad libitum*. Venoms were milked from adult snakes, lyophilized and reconstituted in saline solution prior to use.

Determination of LD_{50} dose

The LD_{50} value was determined according to Karber (1931) and obtained from a dose mortality curve set up especially for venom. LD_{50} was calculated as the aggregate average dose at which the weight 50 % of the animals survived, according to the following equation:

$$LD_{50} = \text{Dose (min)} - \{\text{Sum (Dose (diff) x Average mortality}\}/ K$$

Were K = number of groups.

Surviving animals anesthetized with pentobarbital (60 mg/kg body weight). Dead animals were neglected.

Experimental design

Forty adult male (Age 12-16 weeks) albino rats *Rattus norvegicus* weighing 200–250 g were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH publication No. 85-23. revised in 1985). The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. All animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. Animals were maintained under standard laboratory conditions (temperature 23°C, relative humidity 60-70% and a 12-h light/dark cycle) and were fed a diet of standard commercial pellets and water *ad libitum*.

Animals were equally distributed in two experimental groups. Group I (5 animal), is a control group that was intraperitoneally (*i.p.*) injected with physiological saline (0.2 ml); and group II (35 animals), treated with LD_{50} (0.180 mg/kg, *i.p.*) of the crude *W. aegyptia* venom (ALP) enzyme activity in the liver, abdominal muscles and

cerebrum brain were measured at timed intervals of 1, 3, 6, 12, 24, 72 h and 7 days post envenomation in both the control and treated animals. The histological changes in the liver sections were simultaneously investigated. After scarification, treated animals were immediately anesthetized for twenty minutes and dissected; the liver, abdominal muscles and brain were removed, washed in ice-cold saline, patted dry and weighed. About 100 mg of tissue from the liver, abdominal muscles and cerebrum brain were collected and homogenized in chilled 0.1 M Tris–HCl buffer using Potter–Elvehjem Teflon homogenizer. The homogenates were used for biochemical investigation. Enzyme assay tissues were homogenized in ice cold Tris buffer bath (pH = 7.7) for AST or (pH = 7.4) for ALT and in phosphate buffer (pH = 7.5) for ALP and γ-GT. Determination of the enzymes activity were made according to the recommendations of the Scandinavian Committee on Enzymes (SCE) using Kits from Sera-Pack (Ames Division, Miles Ltd. England) according to manufacturer's instructions. Total protein concentration was determined according to Lowry *et al*. (1951).

Histological examination

A small portion of the liver tissue from the control and treated animals were fixed in 10% neutral buffered formation and then dehydrated with ascending grates of ethanol (70, 80, 90, 95 and 100%). Dehydration was then followed by clearing the tissue samples in 2 changes of chloroform before being impregnated with 2 changes of melted paraffin wax, embedded and blocked out. Tissue section (4-5μm) were stained with haematoxylin-eosin, according to the methods described by Drury *et al*. (1983). Observations and photographs were made using a computerized Olympus optical microscope (Olympus Dp71, Tokyo, Japan).

Statistical analysis

The comparison between the control (group I) and LD_{50} group II, at different time intervals was done using a Student T-test at significance level of $P<0.05$. The data are shown as means±SE and statistically analyzed using SPSS 10 (IBM, USA).

RESULTS

LD_{50} of venom against male albino rat

The LD_{50} value was determined according to Karber (1931). The value of the LD_{50} of W. aegyptia was 0.180/mg/kg mouse body weight (Fig.1).

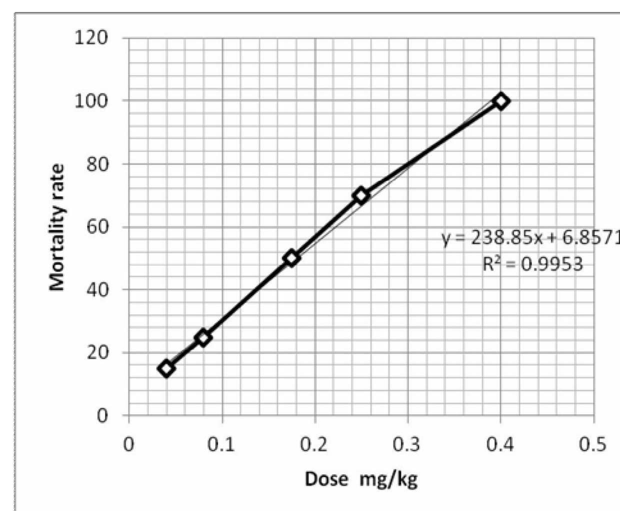

Fig. 1. Twenty four hours dose mortality curve of male albino rats administered different doses of *Walterinnesia aegyptia* venom. The value of the LD_{50} of *W. aegyptia* was 0.180/mg/kg mouse body weight.

Effect of venom on hepatic enzymes

Liver AST was found to be significantly increased 1 h after envenomation but eventually decreased on the 7th day. Also, liver ALT activity was significant increased ($p<0.5$) only at the 1st h and at 24 h. Liver ALT activity decreased below the control at the 7th day of the experiment. Liver γ-GT enzyme activity remained significantly elevated ($P<0.5$) for the first 24 h, then decreased to control level at 72 h and remained at this level until the 7th day (Table I).

ALP activities of tissues

Liver ALP enzyme activity remained close to the control levels for the first 24 h, where upon it significantly increased ($p<0.5$) before returning to the control activity on the 7th day of the experiment. On the other hand, abdominal muscle ALP enzyme activity was found to be significantly decreased at the 1st h, followed by a steadily significant increase

at all time intervals. Brain ALP enzyme activity remained unchanged until the 12th h, then it increased significantly ($p<0.5$). This increase remained significant at 24 and 72 h, but the activity returned to control on the 7th day of the experiment (Table II).

Table I.- Effect of LD_{50} of *Walterinnesia aegyptia* crude venom on hepatic enzymes (AST, ALT and γ-GT) activities of male rats over 7 days.

Group	γ -GT (μ/l) (n=5)	AST (μ/l) (n=5)	ALT(μ/l) (n=5)
Control	0.1±.0.03	5.48±1.4	4.8±1.2
1 h	2.13±0.07*	8.3±0.7*	8.8±1.1*
3 h	3.0±0.02*	7.1±0.5	4.5±20
6 h	1.9±0.03*	5.2±1.3	5.5±1.7
12 h	3.5±0.2*	5.4±1.9	2.8±1.3
24 h	3±.50*	5.4±20	8.3±1.5*
72 h	1.6±.05	5.68±.6	4.4±0.9
7days	1±0.08	4.3±0.4	2.2±0.77

The values are Mean±SE, * significance at < 0.05.

Table II.- Effect of LD_{50} of *Walterinnesia aegyptia* crude venom on the ALP activities in liver, abdominal muscle and brain of male rats over 7 days.

Group	Liver	Abdominal muscle	Brain cerebrum
Control	8.5±1.3	43.5±9.7	831.5± 84.5
1 h	8±2.6	30.4*±6.5*	879±88.5
3 h	7.85±2.1	70.±13.4*	878.7±99.7
6 h	7±1.8	160.4±22.4*	870.8±91
12 h	8.68±2	166±38.9*	602.5 ±64*
24 h	12±1.5*	161.7±33.6*	1007.5±106*
72 h	9.8±0.4	125.6±42.6*	713.75±80.4*
7days	6.65±1.2	64.2±12.9*	798.5±80.6

The values are Mean±SE, * significance at < 0.05.

Total protein content of tissues

Liver protein concentration was significantly increased after 1, 24 and 72 h. While abdominal muscle total protein concentration was significantly decreased continuously from the 1st h to the end of the experiment. There was delayed depletion in brain total protein concentration after 72 h and the 7th day (Table III).

Histological structure of liver

Control liver section (group I) showed central vein (CV) and surrounding hepatocytes (HC), sinusoids (S) lined with van kupffer (K) cells. While group II liver sections (treated with LD_{50} of *W. aegyptia* crude venom)showed inflammatory cellular infiltrations(ICI) around the hepatic vein, dilated blood sinusoids (S), hepatocyticvacuolations (HV) and prominent van kuffer cells (Fig. 2A, B, C and D).

Table III.- Effect of LD_{50} of *Walterinnesia aegyptia*crude venom on the total protein concentration in liver, abdominal muscle and brain of male rats over 7 days.

Group	Liver	Abdominal muscle	Brain cerebrum
Control	6.5±1.2	8.7±1.0	5.5±1.2
1 h	14.2±3.1*	3±1.8*	5.6±1.5
3 h	8.3±2.2	5±1.4*	5.54±1.3
6 h	5.2±1.6	2±0.6*	5.5±1.9
12 h	8.4±2.4	2.3±1.5*	2.9±1.0*
24 h	11.5±2.1*	1.9±0.4*	4±1.4
72 h	11.3±2.6*	4.87±1.7*	2.95±1.3*
7days	7.4±2.3	1.6±0.1*	1.45±0.8*

The values are Mean±SE, * significance at < 0.05.

At 12 h and 24 h, liver sections from the envenomated group showed hepatocytic-vacuolations, inflammatory cellular infiltration and dilated sinusoids. Under higher magnification, marginal chromatin (mc) patterns appear in some hepatocytes and clumped chromatin (cm) in others. With this same group, liver sections taken at 72 h and at 7 days showed increased inflammation and vacuolation as evidenced by an increase in inflammatory cells, some pyknotic cells (Pn), widened sinusoids and numerous van Kupffer cells. Fatty change or Steatosis(St) represents the intracytoplasmic accumulation of triglycerides (neutral fats) of parenchymal organs (Fig. 3A, B, C and D).

DISCUSSION

In the present work, several alteration in the levels of liver enzymes have be detected in envenomated animals throughout the experiment periods. Similar results for liver AST and ALT levels have been found to be fluctuated after

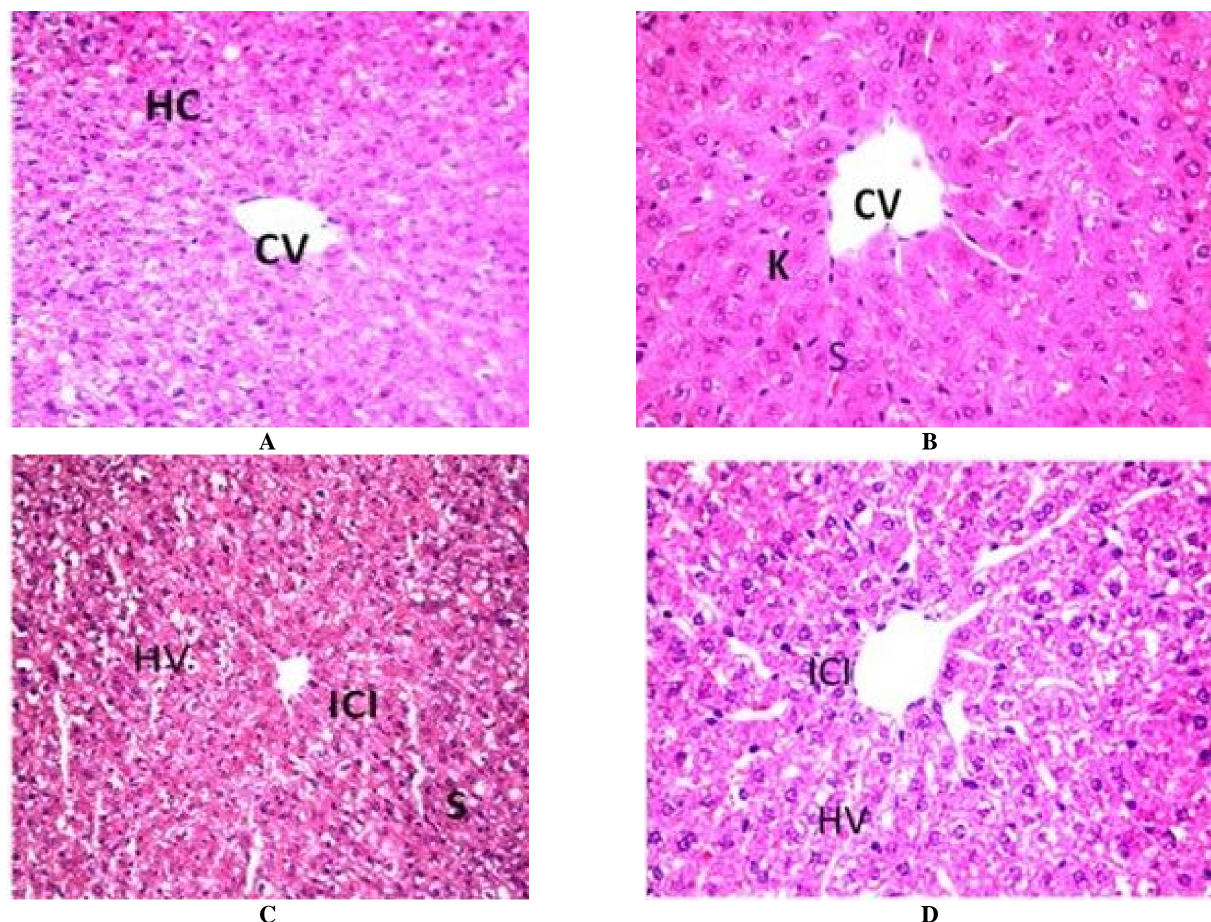

Fig. 2. Histological changes induced by treatment with LD_{50} of *Walterinnesia aegyptia* crude venom in liver 3 to 6 h post envenomation.
Control liver section showed central vein (CV) and surrounding hepatocytes(HC), (Fig. 2A). sinusoids (S) lined with van kupffer (K) cells (Fig. 2B). While *W. aegyptia*group liver sections (taken at 3h and 6 h post envenomation showed inflammatory cellular infiltrations(ICI) around the hepatic vein, dilated blood sinusoids (S) (Fig. 2C)., hepatocyticvacuolations (HV) and prominent van kuffer cells (Fig. 2D). (H& E stain, X40 and X100).

different types of snake's envenomations (Mohamed *et al*., 1981; Assi and Naser, 1999), revealing symptoms similar to hepatitis, liver cirrhosis and muscular dystrophy (Murray *et al*., 1988; Porth, 1990). Mohamed *et al*. (1981) reported that *Naja haje* venom induced a significant increase in liver AST activity which may be due to destruction of hepatic cellular organelles and intracellular liberation of these enzymes. Decrease in the levels of liver ALT could be explained by the glucose-alanine cycle in which pyruvate produced from glucose is transaminated to alanine via ALT enzyme and transported to liver to be reconverted to glucose by gluconeogenesis to enhance the hyperglycemic phenomenon observed after envenomation (Felig, 1975). The possible utilization of ALT in this mechanism could be the reason for its low level observed in the current study, taking into consideration that protein content was increased in liver and the amino acids of these proteins may act as a pool undergoing the transamination processes and the de novo synthesis in the liver.

γ-GT enzyme is produced in many tissues including those of liver. Our data revealed an elevation in the serum γ-GT level, especially along with elevations in alkaline phosphatase as described

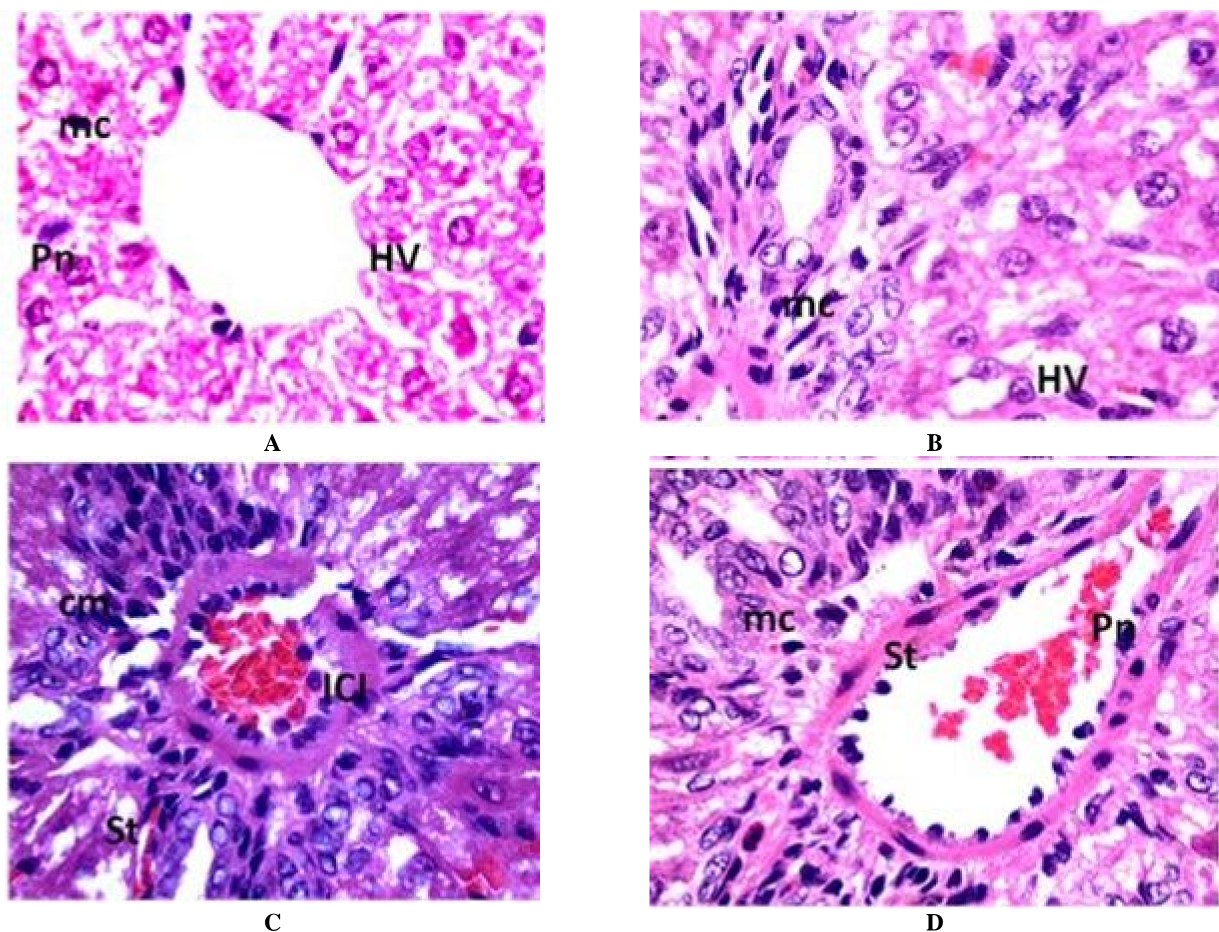

Fig.3. Histological changes induced by treatment with LD_{50} of *Walterinnesia aegyptia* crude venom in liver 12, 24, 72 h to 7 days post envenomation.

At 12 and 24 h liver sections from the envenomated group, hepatocyticvacuolations, inflammatory cellular infiltration and dilated sinusoids showed. Under higher magnification, marginal chromatin (mc) patterns appear in some hepatocytes and clumped chromatin (cm) in others (Fig. 3A). With this same group, liver sections taken at 72h and at 7 days showed increased inflammation and vacuolation as evidenced by an increase in inflammatory cells (Fig.3B)., some pyknotic cells (Pn), widened sinusoids and numerous van Kupffer cells (Fig. 3C). Fatty change or Steatosis(St) represents the intracytoplasmicaccumulation of triglycerides (neutral fats) of parenchymal organs (Fig.3D) (H& E stain, X40 and X100).

in our previous study (Al-Sadoon *et al*., 2011), suggest a damage in the bile duct structures. Increases in lesions cause intra hepatic or extra hepatic obstruction of bile ducts, including parenchymatous liver diseases with major cholestatic hepatitis. These pathological changes reflect altered metabolic processes since most enzymes are present in cells at much higher levels than in plasma. However, normal plasma enzyme level reflects the balance between the release of enzyme during ordinary cell turnover and their metabolism and excretion. Increase in the levels of certain enzymes such as ALP and γ-GT is considered as a marker for cell damage and degeneration. On the other hand a decrease in the levels of these enzymes indicates a reduced synthesis, direct inhibition or failure to be excreted due to cell damage by the toxic venom (Mohamed *et al*., 1981). ALP enzyme is found mainly in the bile ducts of liver and increase in its level can indicate an obstructive or cholestatic liver disease. The present data demonstrated that the activity of ALP

enzyme in liver; abdominal muscles and brain has been fluctuated over 7 days. Elevation in the activity of ALP in liver in animals treated with *W. aegyptia* venom gives additional evidence of liver damage. Similar results have been previously reported (Ueno and Rosenberg, 1996). Alteration in the activity of ALP enzyme in the brain tissue as mentioned in this study could be due to the neurotoxic effects of the Elapidae venom as reported by Ismail and Abd-Elsalam (1988) and AL-Jammaz *et al*. (1992).

In the present study, histological examination of liver sections taken from envenomated animals demonstrated pyknotic nuclei, clumped chromatin and inflammatory cellular infiltrations. Similar results have reported by Mohamed *et al*. (1978) who used LD_{50} of *N. haje* venom.

Significant increase was detected in liver protein content after 1, 24 and 72 h, but it decreased to normal level at 7th day of the experiment. Rahmy and Hemmaid (2000) observed that an injected sub-lethal intramuscular dose of *N. haje* venom caused alterations in liver total protein. Significant depletion in the total protein concentration of abdominal muscles was continuous from the 1st h to the end of the experiment but depletion in brain total protein was delayed until 72 h. This could be explained on the basis that the snake venoms contain several proteolytic enzymes and proteins with different toxicological functions and special pharmacological effects (El-Refael and Sarkar, 2009; Evangelista *et al*., 2010). The disturbance in protein synthesis in the hepatocytes could be due to cellular damage. The venom directly affects the abdominal muscles in a step of its digestion process, and to a lesser extent the brain as the nervous system is known to be more resistant to toxins effects. The continuity of protein degradation in abdominal muscle studied could not be attributed only to the proteolytic effect of venom as its concentration decline with time, but it could also be a direct effect of insulin deficiency noted after envenomation (El-Refael and Sarkar, 2009) and hence causing a diabetogenic effect. Also it is possible that the venom altered the gluconeogenesis mechanism, especially in liver and kidney - favoring the usage of the key amino acids and resulting in the augmentation of serum glucose level (Fahim, 2001). Inflammation and vacuolation, some pyknotic cells as well as fatty change or steatosis (St) represents the intra cytoplasmic accumulation of triglycerides (neutral fats) as observed in liver sections are possible effects of the *W. aegyptia* venom. Phospholipases A2 (PLA2) specifically catalyzes hydrolysis of the sn-2 ester bond in glycerophospholipids to give lysophospholipids and fatty acids. GIsPLA2s are found in venoms of Elapidae (Sajevic *et al*., 2011). In summary, the process of physiological adaptation and recovery from LD_{50} of *W. aegyptia* crude venom seems to be stabilized after one week, leaving the animal alive with several lesions and disturbed physiological profile, especially in liver and muscles.

ACKNOWLEDGEMENTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no**.** RGP-VPP-289". We wish to express our deep thanks to Dr. Syfer for reading the manuscript and to Mr. Tulba and Mr. Al-Otabi H. for technical assistance.

The authors declare no conflict of interest.

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(Received 25 May 2013, revised 15 June 2013)