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Determination of *Naja haje* crude venom LD50 and the study of its nephrotoxic effect in Wistar rat.

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Abstract: Nephrotoxicity is a common sign of snake poisoning. The present work aims to clarify the effect of intramuscular injection of LD50 and 1/2 LD50 dose (about 0.25 mg/kg of mice) of N. haje venom on the kidneys of mice after 3, 6, and 9 hours of poisoning, respectively. Histopathological changes appeared after 3 hours of injecting the venom for each of LD50&1\2LD50. They were recorded in the form of tubular damage, glomerulosclerosis changes, vascular congestion, focal infections for all groups of N. haje crude venom and it was noted that the severity of these alterations increased in a dose and time-dependent manner. Moreover, focal fibrosis appeared with LD50 at 6,9 hours. It can be concluded that the onset of changes was in the first 3 hours for different venom groups, but with higher doses was more severe. Our findings confirm that acute exposure to N. haje crude venom causes nephrotoxicity in rats.

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Key words: *Naja haje* crude venom, LD50, nephrotoxicity, rat.

1- Introduction

Snakes are important members of class Reptiles. Snakebites are common throughout the world and about 5.5 million people are bitten by snakes annually all over the world (Ebaid et al., 2007) Several species of cobras are natives of Africa, among them is the Egyptian cobra *Naja haje* (Linnaeus) found from southern Egypt to northern South Africa (Binh et al, 2010). The toxin is composed of polypeptides [Hider et al., 1991]. Purines, amines, lipids, organic molecules, metal ions, carbohydrates, and lipids (Aird, 2002) non-enzymatic polypeptide toxins neurotoxins and cardiotoxins), and other substances (Ponnappa et al., 2008). Envenomation causes local pain and swelling and may be associated with blistering at the bite site. Neurotoxic and systemic symptoms develop within a few hours, and deaths have occurred within 6–16 h after large snake bites, despite the use of anti-venom and mechanical ventilation. Histological, histochemical, and biochemical fluctuations triggered by the venom of the Egyptian cobra (*Naja haje*) have already been evaluated in rodent animals (El-Fiky, 1999; Omran et al., 1997). In severe cases, tissue damage reaches many organs, such as the brain, lung, kidney, heart, and liver (Lipps, 2001; Mukherjee and Maity, 2002). In a previous study, a sub-lethal dose of the Egyptian cobra venom was found to induce a deleterious action on the histological and histochemical of animal renal tissues, was previously reported by (Gunatilake et al., 2003; Sitprija, 2206). In the study of (Ahmed et al., 2015). Rats were injected with venom (0.25 mg/kg) there was oxidative/nitrosative damage and apoptosis in the liver, heart, and kidneys of venom-injected rats. (Kamel, 2010) Recorded that, the histopathological and ultrastructural changes occurring in the renal cortical cells of rats following intramuscular injection of ⅛ LD**50** (0.0625 mg/kg body weight) of cobra (*Naja haje*) crude venom, at 4 h, 8 h, and 12 h time intervals following injection of the venom.

Overall, the factors associated with sAKI varied across studies due to differences in the study population, potency, and composition of snake venom, which differs across geographic regions of the study sites; accessibility of management facilities; and study design. (Albuquerque et al., 2014; Sarkar et al., 2018).

Kidney failure caused by snake venom is still a public health problem, especially in rural areas, and most victims cannot be dealt with at the beginning of the injury, and often deaths occur before the victims reach the hospital (Naqvi, 2016).

Timing and dosing of the venom appear to be very important for treatment use after bites and early intervention before tissue damage develops, Whereas, delaying the presentation of ASV or waiting for the victim to show systemic manifestations, meaning that

waiting for 6 hours results in systemic poisoning and high mortality,

Therefore, more studies are needed to provide additional data on geographical differences between these areas and to improve the ability of health care workers to help victims In a previous study, a sub-lethal dose of the Egyptian cobra venom and other species of the cobra was found to induce include acute kidney injury, hematological manifestations, and other organ involvement (Felipe Silva et al., 2019; Taha et al., 2015, Riaz et al., 2015; Rania et al., 2015; Al-Mamun et al., 2015).

Through these studies, we have found that few are conducted on histopathological effects at ld50 doses of N. haje venom the snake, which is one of our most common species. Thus, The present study aimed to evaluate nephrotoxicity of intramuscular injection of LD50 and 1/2 LD50 doses of *Naja haje* crude venom on the renal of rats after 3, 6, and 9 hours from envenomation This was determined by histological examination of kidney tissues.

2. Materials and Methods

Materials

2.1 Animals

Ninty adult male rats body weight of (200 ± 20g). The rats were maintained under control room temperature of 22 ± 3C0 with 12 hours light/dark cycles and the humidity level of 50- 60%, All animals had access to laboratory Standard feed and tap water.

2.2 Venom

Lyophilized *Naja haja* venom was obtained from Egypt (Center of serum and vaccine in Alexandria). Lyophilized venom was 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) were obtained from Sigma.

2.3 Determination of LD50 dose

LD**50** of crude venom determine as describe by (Weil, 1952). 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, space the dosage levels so that they are in a geometric progression. Mortality rates (f), 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 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 (Weil, 1952)

The LD**50** toxicity values of collecting the Egyptian cobra ***Naja haja*** were assessed by injected (i.m) rats and Calculated by:

$$Logm=logD+d(f+1)$$

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

Our results are consistent with other studies on the value of LD**50** of crude venom of the Egyptian cobra 9Esmat et al., 2003; 24, Ernst and Zug,1996; EL-aal and Ezzat,1997, Ezzat and Abd EL-aal, 1989; Ahmed and Abdel Moneim,2015; Taha,2015)

2.4 Experimental design

Animals were divided into three experimental groups:

Control group: Rats intramuscularly (i.m.) injected only with 0.1 ml phosphate buffered saline (PBS) without venom, and was killed after 9 hours of the injection.

LD50-Envenome group: Rats i.m single does inject with 0.1ml phosphate buffered saline (PBS) containing LD**50** (0.25mg cobra venom / kg body weight of the rat). The rats were subdivided into three subgroups (ten rats each) was killed after 3, 6 and 9 hours from envenoming respectively.

½LD50-Envenome group: Rats i.m single does inject with 0.1ml phosphate buffered saline (PBS).Containing ½LD**50** (0.125mg cobra venom /kg body weight of the rat). This group subdivided into three subgroups (ten rats each) was killed after 3, 6 and 9 hours from envenoming respectively. (Lougin et al., 2001)

2.5 Histological studies

Autopsy samples were taken from the kidney of rats in different groups and fixed in 10% Formalin saline for twenty-four hours. Washing was done with tap water, then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration, the alcohol concentrations used for dehydration were 30%, 50%, 70%, 90% and 100%. Specimens were cleared in xylene and embedded in paraffin at 56 degrees in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin &eosin stain and cover slides with a glass cover using a Canada balsam for examination through the light electric microscope (Leica ICC50HD), (Bancroft and Stevens,1996). Pictures were taken using a microscope equipped with a machine A digital photograph, model (GmbH, CMS Microsystem Leica).

3. Results

Control groups

There was no histopathological alteration and the normal histological structure of the glomeruli and tubules at the cortex as well as the tubules at the corticomedullary portion were recorded in control groups (Fig.1, 2).

As for the treated groups,The first appearance of the changes was at a time of 3 hours for both doses with a difference in the severity of the changes. It was more severe with the higher dose and more severe with increasing time.

LD_{50} groups of the Egyptian cobra *Naja haja* venom and killed after 3, 6 and 9 hours.

There were periglomerular as well as perivascular and inter tubular inflammatory cells aggregation observed after 3h of injection (Fig.3&4). The cortical blood vessels showed sever congestion. The corticomedullary portion showed also focal inflammatory cells aggregation between the tubules.

Focal inflammatory cells aggregations were detected in the periglomerular tissue as well as surrounding the congested blood vessels at the cortex after 6h of injection (Fig.5). The corticomedullary portion showed focal fibrosis in between the atrophied tubules.

Sever congestion was detected in the cortical blood vessels associated with periglomerular inflammatory cells infiltration after 9h of injection (Fig.6). The perivascular tissue also showed inflammatory cells aggregation (Fig.7). The corticomedulary portion showed focal fibrosis in between the atrophied tubules (Fig.8).

$_{1/2}LD_{50}$ group of the Egyptian cobra *Naja haja* venom and killed after3,6 and 9 hours.

Sever congestion was noticed in the cortical blood vessels and glomerular tufts after 3h of injection.

There was focal inflammatory cells aggregation in the periglomerular as well as the perivascular congested blood vessels at the cortex after 6h of injection (Fig.9).Also degenerative change was detected in the lining epithelium of the tubules at the corticomedullary protion (Fig. 10).

Focal inflammatory cells aggregation was detected in between the tubules as wellasin the periglomerular tissue at the cortex after 9h of injection. There was perivascular inflammatory cells aggregation and at the cortex, The corticomedullary portion showed focal an inflammatory cells aggregation between the tubules (Fig.11).

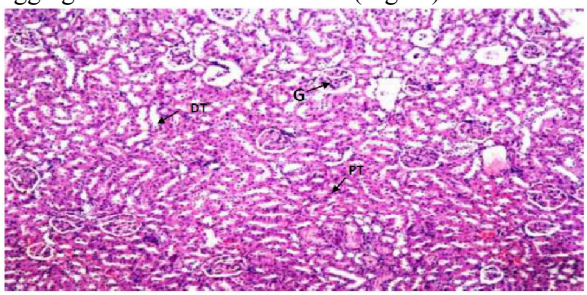

Fig (1) kidney section of control Rat showing normal histological structure of glomeruli (G) and tubules (proximal tubule PT, distal tubules DT) at the cortex. (H&E, x16).

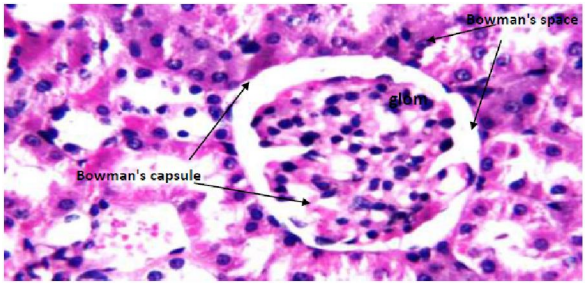

Fig (2) kidney section of control Rat showing normal histological structure of glomeruli, glomerulus (glom). Bowman' s capsule and Bowman' s space (H&E, x40).

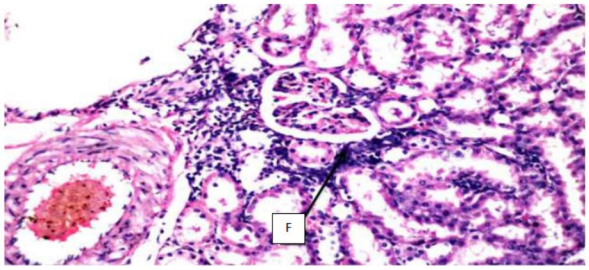

Fig (3) kidney section of Rat after 3 hours from injection of the Egyptian Cobra crude venom at (LD_{50}) showing periglomerular inflammatory (F) aggregation. (H&E, x40).

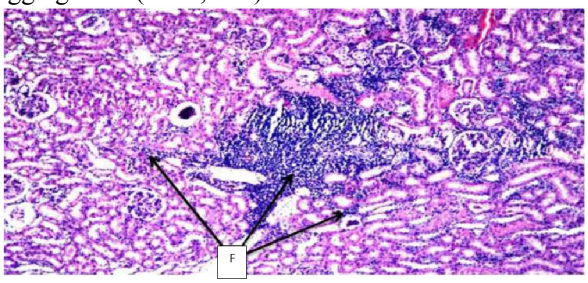

Fig (4) kidney section of Rat after 3 hours from injection of the Egyptian Cobra crude venom at (LD_{50}) showing focal inflammatory cell (F) aggregation between tubules at the cortex. (H&E,x)

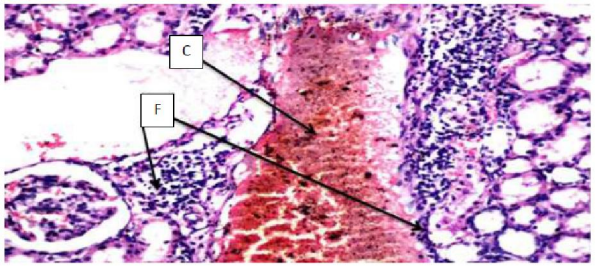

Fig (5) kidney section of Rat after 6 hours from injection of the Egyptian Cobra crude venom at (LD_{50}) showing focal aggregations of inflammatory cell (F) in periglomerular tissue and surrounding the

congested (C) blood vessels at the cortex. (H&E, x40).

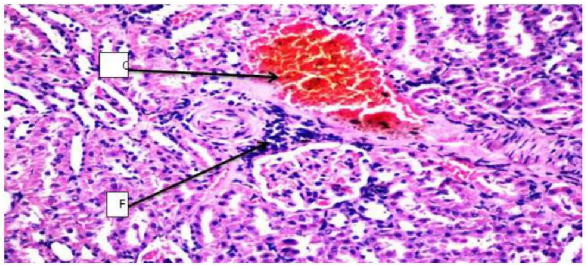

Fig (6) kidney section of Rat after 9 hours from injection of the Egyptian Cobra crude venom at (LD_{50}) showing sever congestion (C) in cortical blood vessels with periglomerular inflammatory cells (F) infiltration. (H&E, x40).

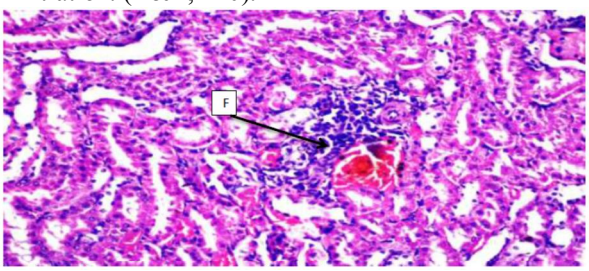

Fig (7) kidney section of Rat after 9 hours from injection of Egyptian Cobra venom crude at (LD_{50}) **showing** perivascular inflammatory cell (F) aggregation at corticomedulary portion. (H&E,x40).

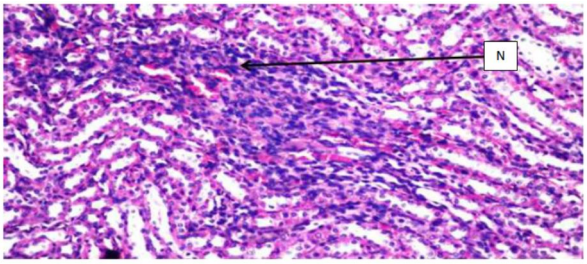

Fig (8) kidney section of Rat after 9 hours from injection of the Egyptian Cobra crude venom at (LD_{50}) showing focal fibrosis (N) between the atrophied tubular at corticomedulary portion. (H&E, x40).

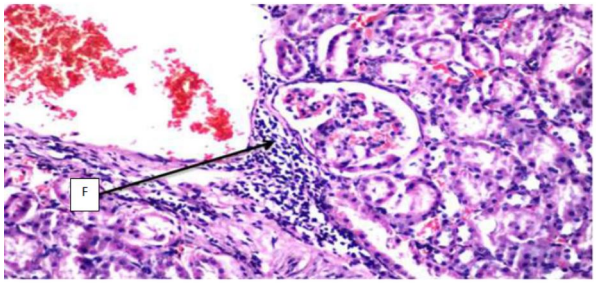

Fig (9) kidney section of Rat after 6 hours from injection of the Egyptian Cobra crude venom at ($½LD_{50}$) showing to identify the periglomerular inflammatory cell (F) aggregation.(H&E,x80).

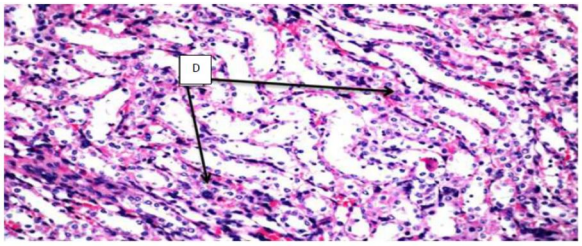

Fig (10) kidney section of Rat after 6 hours from injection of the Egyptian Cobra crude venom at ($½LD_{50}$) showing Degenerative (D) in tubules lining epithelium at the corticomedullary protein (H&E, x40).

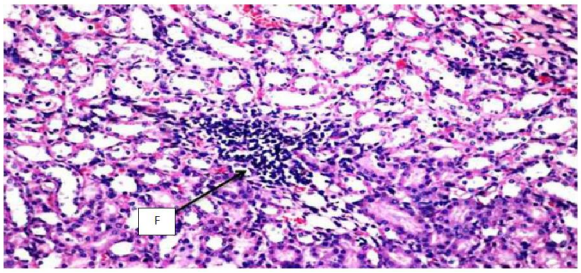

Fig (11) kidney section of Rat after 9 hours from injection of the Egyptian Cobra crude venom at ($½LD_{50}$) showing focal inflammatory cells (F) aggregation in between the tubules at corticomedullary portion. (H&E, x40)

4. Discussion

Snakebite envenomation is one of the most important, although neglected, health problems all over the world, particularly in Africa, Asia, and Latin America (Gutierrez, 2006; WHO, 2007; Alirol et al., 2010).

The commonest important systemic manifestations of snake envenoming are venom-induced consumption coagulopathy, neuromuscular paralysis, acute kidney injury, myotoxicity, and cardiovascular collapse (Gutiérrez et al., 2008; Taha., 2015; Ranjith et al.,2020). Several works dealing with the effects of snake venom in blood cells, marrow cells, and in cells from other organs of animals, like muscle, liver, kidney, and skin, showed varying results, depending on the experimental concentrations, exposure time, site of injection, and type of toxin(Fox and Serrano, 2008; Tohamy et al.,2014).

Several works dealing with the effects of snake venom in blood cells, marrow cells, and in cells from other organs of animals, like muscle, liver, kidney, and skin, showed varying results, depending on the experimental concentrations, exposure time, site of injection, and type of toxin(Fox and Serrano, 2008; Tohamy et al.,2014)]. The kidney, a highly

vascularized organ to excretory function, is prone to venom toxicity as an innocent bystander. AKI, the most significant of all the renal manifestations, has been reported with varying frequency in different studies (Lipps, 2001). Many studies have appeared in the literature on snake bite-induced AKI (Natarajan, 2019; Wei et al., 2016; Myanmar, 2017).

Imam and Rahmy found that the dose induces a change in sub-lethal doses of *N. haje* venom-induced histopathological, histochemical, and pathophysiological alterations in the heart, liver, kidney, and brain of rats (Imam and Rahmy, 2001).

After 3 h of envenoming with ½LD50 dose, the renal tissues revealed severe congestion was noticed in the cortical blood vessels and glomerular tufts, while focal inflammatory cells aggregation in the periglomerular, as well as the perivascular congested blood vessels at the cortex and degenerative change, was detected in the lining epithelium of the tubules at the corticomedullary junction were noticed at 6 hr, were increasingly extended by 9hours of envenoming. On the other hand, with an injection of the LD50 dose, the alterations were more severe and early onset compared with ½LD50, after 3 hr. of envenoming, focal inflammatory cells were common in many renal tissues, and alterations were increasingly extended by 6 and 9 h of envenoming with the appearance of focal fibrosis in between the atrophied tubules. This finding is by the previous studies of (Al-Mamun et al., 2015, Tohamy et.al, 2014).

The risks of acute nephrotoxicity of snake venom appear 2 hours after the venom is injected (Albuquerque et al., 2014, Athappan et al.,2008) The incidence of tubular cell necrosis and the degeneration of most cellular organelles increases 6 to 12 hours after the venom is injected This was explained by (Farías et al.,2012). As they suggested that free fatty acids are directly responsible for the observed effects induced by phospholipase A2 and In Russell's viper envenomation they found that with an increased time interval after envenomation, the activities of all lysosomal enzymes generally increased, and the lysosomal membrane integrity was reduced. This could indicate a defensive action of renal cells during the progression of venom toxicity (Rahmy et al., 1992). This action was attributed to the effect of different venom toxins, such as myotoxins, cytotoxins, phospholipases, and cardiotoxins (Rahmy and Hemmaid, 2001). It was found that myotoxin probably causes renal damage due to myoglobin cast nephropathy. Venom phospholipase is known to be toxic to cells and is believed to be responsible for disturbing the cell membrane permeability (Dkhil et al., 2014). Phospholipase A2 can cause membrane injury and tubular necrosis (Kini, 2003).

Amany *et al*., 2014 and (Wang et al., 2000), Indicated that inflammatory cellular infiltration, vacuolation in the tubule, and shrinkage of glomeruli in most cases in the renal structure of envenoming mice injected with 1/2LD50 *Naja haje* venom. According to (Gunatilake et al., 2003). A sub-lethal dose of the cobra venom was found to effects the histological and histochemical patterns of the renal tissue of rabbits including complete necrosis in the glomeruli and proximal and distal convoluted tubular cells. Renal tissue of envenomated animals showed tubular lesions with subsequent accumulation of inflammatory cells in the tubular tissues probably due to the recovery of the injury by cobra itself. (Ebaid et al., 2007) The target-specific PLA2 is enzymes, which cause hemorrhage in the lung, liver, pituitary, thyroid, and kidney, which have been isolated and characterized from Russell's viper venom (Vishwanath, 1986). The indirect action might be due to the deadly effect brought about by reactive metabolites in the kidney during envenoming (Niesink et al.,) so; Oxidative stress may be a result of excessive reactive oxygen species generation or failure of the cellular antioxidant system. Snake venom-induced an elevation of oxidative stress indicators as nitric oxide, lipid peroxidation (Asmari et al., 2006), and glutathione (Mitchell and Jollow, 1975) and therefore leading to cell damage (Wang, 2000). Conclusion: From these observations, we can be concluded that N. haje crude venom causes acute nephrotoxicity, And that appeared after 3 h of envenoming with LD50 and ½LD50 dose, The changes develop over time and are more severe with a higher dose. The timing and dosing of the toxin appear to be very important for the use of post-exposure therapy and early intervention before tissue damage occurs, In the future, we look forward to studying the mechanisms responsible for this process, and by which appropriate treatment can be found.

5. Compliance with Ethical Standards

All experiments, transportation, and animal care used in this study were following the College of Science regarding research ethics and animal handling. The tests were also conducted by the conditions of safety and laboratory safety.

6. Acknowledgments

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