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Toxicopathological Effects of the Venom of *Echis ocellatus* on Experimentally Envenomated Swiss Albino Rats

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ABSTRACT: The World Health Organization classified snakebite envenomation as one of the Neglected Tropical Diseases in 2017. The significance of *Echis ocellatus* in causing envenomation in humans and animals in Northern Nigeria has been widely recognized, with experts identifying it as the most medically important snake in the region. Consequently, this study aimed to investigate the toxicopathological effects of *Echis ocellatus* venom on Swiss albino rats subjected to experimental envenomation. The vital organs were collected and examined histopathologically. The data revealed necrosis and congestion in the heart tissues of envenomated rats compared to the control group. Similarly, the kidney sections showed necrosis and congestion in the nephrotic tissues, while the liver sections exhibited necrosis, congestion, and perivascular cuffing. Data obtained show that the haematological parameters: PCV was 26 ± 0.7723% in the envenomated group compared to 44 ± 0.5213% in the control group. Hemoglobin levels were 8.67 ± 0.4217 g/dL versus 14.23 ± 0.3871 g/dL, erythrocyte counts were 1.0 ± 0.5617 $\times 10^6$/L compared to 9.0 ± 0.3938 $\times 10^6$/L, and total protein was 3.2 ± 1.1623 g/dL compared to 8.6 ± 0.2928 g/dL in the controls. MCH was 21.675 pg compared to 15.556 pg in the controls. Table 2 provides leukocyte parameters: leukocyte counts were 12.0 ± 0.5429 $\times 10^9$/L for the envenomated group and 12.12 ± 0.2419 $\times 10^9$/L for controls. Monocyte levels were 0.0 ± 0.00% in the envenomated group versus 1.6 ± 0.2844% in the controls. These findings suggest the venom of *Echis ocellatus* induces significant hematotoxicity, with pathological involvement of the heart, liver, kidneys, and spleen.

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Snake venoms are a specialized cocktail of polypeptides (Lee, 2009) which are toxins that are enzymatic or non-enzymatic. Other snake venom components include carbohydrates, lipids, nucleosides, biogenic amines, proteins and metals (magnesium, calcium, and zinc) (Koh *et al.*, 2006). Usually, snake venoms are classified based on their modes of action. The different types so far

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documented are described as haemotoxic, neurotoxic, cytotoxic, myotoxic, nephrotoxic or cardiotoxic (Theakston *et al.,* 2003; Yusuf *et al,* 2015; Ameh *et al.,* 2019). Snake venom has also been implicated in cardiac muscle necrosis leading to cardiotoxicity associated with snake envenomation (Theakston *et al.,* 2003[a]; 2003[b]; EchiTab, 2008; Yusuf *et al.,* 2015). That feature has been reported in most classes of venomous snakes (Koh *et al,* 2006; Mackessy, 2009; Kini and Doley, 2010). Several snakes have been reported to cause envenoming in Northern Nigeria (Habib, 2013, Abubakar *et al*., 2010, Yusuf *et al*., 2015), *Echis ocellatus* is believed to be responsible for most bites in Northern Nigeria (WHO, 2010, Abubakar *et al*., 2010). *Echis ocellatus* is believed to be responsible for more snakebite envenoming in Northern Nigeria than all other species put together (Yusuf *et al*., 2019). Although most reported cases of snakebite in Northern Nigeria occur in humans (with very few exceptions), snake envenomation of domestic animals occurs frequently, has a worldwide distribution, and has been reported in dogs, cats, horses, sheep, cattle, and New World camelids (Goddard *et al.,* 2018, Willey *et al.,* 2005, Anlén 2008, Dykgraaf *et al.,* 2006, Yusuf *et al.,* 2019). The existing protocol for the management of snake envenoming by the WHO was purely designed based on human encounters. This present work therefore focused on the clinic-pathologic effects of the venom of snakes of Northern Nigeria in Swiss albino rats with the intent of proffering suggestions that can be more easily extrapolated for management of snake envenoming for veterinary use.

MATERIALS AND METHODS

Ethical Approval: Ethical approval was sought from the animal use and ethics committee of Ahmadu Bello University, Zaria, Nigeria.

Median lethal dose (LD_{50}): The LD_{50} was determined using the up-and-down method described by Bruce (1987) and Depass (1989). In this method, animals are dosed one at a time starting at an estimated LD_{50} dose. If the first animal survives, the next animal receives a higher dose. If the first animal dies, the next animal receives a lower dose. Doses are usually adjusted by a constant multiplicative factor such as 1.3. The dose for each successive animal is adjusted up or down depending on the outcome of the previous animal.

In vivo inoculation: Adult male albino rats weighing 95 – 120g were used for this study. Ten rats were randomly divided into 2 groups of 5 rats per group and labelled A and B. Mice were provided with feed and water *ad libitum*. Feed was compounded from commercial poultry feed. Mice were sourced from the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. They were allowed to acclimatize to the animal room of the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, for 2 weeks. Twice the LD_{50} of crude venom of *Echis ocellatus* (5.64 mg/kg) was injected intraperitoneally into rats in group A (Dong *et al,* 2004). Group B was injected with distilled water. Feed and water were provided *ad libitum* and clinical signs of envenomation were recorded. Rats were observed for 24 hours after which 1ml of blood was taken from each surviving mouse in 3.8% sodium citrate for haematological evaluation. The rats were then sacrificed and the heart, liver, kidneys, spleen, lungs and brains were harvested and stored in 10% formalin for histopathological examination.

Evaluation of packed cell volume (PVC): The packed cell volume (PCV) was determined using the standard technique as described by Rehman *et al*. (2003). The non-heparinized capillary tube was filled up to about ¾ of its length from one end and the second end was heat-sealed using a Bunsen burner. The blood in the sealed capillary tube was then centrifuged for 5 minutes at 4,383 x *g* using the Saitexiangyi TG12MX® Micro-haematocrit centrifuge machine. Then the proportion of cells in the total volume of blood was measured and recorded as a percentage using the Hawksley® Micro-haematocrit Reader.

Evaluation of erythrocyte and total leukocyte counts: Red blood cells (RBC) and total WBC (or TWBC) counts were determined as described by Natt and Herrick (1952) as modified by Campbell and Ellis (2007) using the Natt-Herrick solution (1:200 dilution) and the Improved Neubauer haemocytometer as both counts can be prepared directly from the same sample placed in the haemocytometer. The heparinised blood samples were slightly agitated and the RBC diluting pipette was used to pipette the blood to the 0.5 markings. The tip of the pipette was cleaned properly using tissue paper without touching the distal opening of the pipette tip with tissue, as this will cause a capillary shift of blood into the tissue. The diluting solution (Natt-Herrick) was also pipette to the 101 markings (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was well shaken for 1 minute to obtain equal distribution then emptied into a clean sample bottle. The Neubauer haemocytometer and coverslip were cleaned using a dry, lint-free cloth. The coverslip was properly placed on the haemocytometer. The mixture was then agitated a little and a capillary tube was used to withdraw a

small aliquot. One side of the haemocytometer was filled up (charged) by gently touching the intersection between the coverslip and haemocytometer with the loaded capillary tube avoiding air bubbles and under-filling or over-filling, then left for 5 minutes for cells to settle down.

The light microscope (Olympus-XSZ-107BN), at low power magnification (X40), was used to view the cells and counting was done using the tally counter.

For TWBC count, the WBC in the four outer large squares of the haemocytometer were counted and calculated using the formula below:

$$\frac{N}{20} = WBC\ x\ 10^9/L$$

Where N = Number of WBC counted in the four outer large squares (or in 64 small squares)
For RBC count, the cells contained in the four corners and central squares in the mid-section of the haemocytometer were counted. Following the "**L**" rule: cells that touch the centre triple lines of the ruling on the left and the bottom sides were counted but cells that touch the centre triple lines of the ruling on the right and the top sides were not counted. The RBC count was calculated using the formula below:

$$\frac{N}{100} = RBC\ x\ 10^{12}/L$$

Where N = Number of RBC counted in the 5 squares in the mid-section of the haemocytometer (or in 160 squares)

Determination of differential leukocyte count: A small drop (about 2 µL) of blood was immediately used for the preparation of blood smears each using the standard slide-to-slide technique. The air-dried smears were properly labelled using a pencil on the frosted end of the slide and then fixed in a fixing jar containing methanol for 3 minutes and air-dried. Staining was done by flooding the smears with Wright-Giemsa stain for 3 minutes. An equal amount of Sørensen's buffer (pH of 6.8) was added then mixed gently by blowing using a pipette until green metallic sheen forms on the surface. This was allowed to stand for a further 6 minutes. The smears were rinsed with the Sørensen's buffer and allowed to stand for a minute for differentiation. The stained slides were then washed copiously with Sørensen's buffer and the back of the smears were wiped with tissue paper to remove the excess stain and allowed to air dry. These were neatly packed into a slide box until viewing. Examination of the blood smears was done using a light microscope (Olympus-XSZ-107BN) under high-power magnification with oil immersion (X1,000). One hundred WBCs were counted and classified based on their morphologic features (Campbell and Ellis, 2007). The counting was done using the Marble® Blood Cell Calculator. The differential WBC count was then expressed as a percentage of the individual cell group. The percentage of each cell was then converted into absolute numbers by reference to the total WBC using the formula below:

$$\frac{Percentage\ of\ WBC\ counted\ x\ TWBC}{100} = Absolute\ Number\ x\ 10^9/L$$

Determination of haemoglobin concentration: Blood haemoglobin concentration was assayed colourimetrically as cyanomethhaemoglobin (Drabkin, 1945). Five millilitres of HICN (Drabkin) solution were measured using a 5 ml syringe into plastic test tubes. Twenty microlitres (20 µL) of blood were measured using a micropipette and added to the Drabkin solution in the test tube and properly mixed by gently shaking the test tube. It was centrifuged at 1,500 x *g* for 10 minutes to separate the empty RBC from interfering with the reading. The supernatant was separated into a sample bottle. The mixture was absorbed into the haemoglobin meter (XF–C, China). After the wiggling pump stops working, the value displayed on the screen was recorded in g/dL as the haemoglobin concentration.

Evaluation of erythrocytic indices: The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were obtained as described by Campbell and Ellis (2007), as follows:

MCV = (PCV * 10)/Erythrocyte = MCV (fl)

MCH = (Hb * 10)/Erythrocyte = MCH (pg)

MCHC = (Hb * 100)/PCV = MCHC (g/L)

Histopathologic Examination: Tissues harvested were preserved in 10% buffered neutral formalin and were processed for histopathological examination using standard histology technique (Luna, 1968) in the Histopathology Laboratory of the Department of Veterinary Pathology, Ahmadu Bello University, Zaria. The tissues were dehydrated in ascending grades of alcohol (70%, 80%, 90% and absolute), cleared in xylene and embedded in paraffin wax. Thereafter, they were blocked, sectioned at 5 µ thickness and stained with haematoxylin and eosin (H&E). Slides were viewed using a light microscope

at different magnifications (x40, x100, x200 and x400) and the pathologies were recorded.

Data analyses: Data obtained from the study were expressed as mean ± standard error of the mean (Mean ± SEM). Values were subjected to a one-way analysis of variance (ANOVA). Data were analysed using GraphPad Prism 6.0 Software for Windows (GraphPad Software, San Diego, California, USA). Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The LD_{50} was calculated as 2.82 mg/kg. This is in the same range as the Australian Venom and Toxin Database (2006), where the LD_{50} of *Echis ocellatus was* calculated to be 2.71 mg/kg by intravenous route of administration. Following administration of the venom of *Echis ocellatus* in Swiss albino rats, clinical signs manifested were stretching, restlessness, aggression, shivering, rough hair coats and dry gangrene at the site of injection. The rats were all depressed. Clinical manifestations of *Echis ocellatus* were suggestive of pains, necrosis and haematological crisis. EchiTAb (2008), Abubakar *et al.* (2010), Yusuf *et al.* (2015), and WHO (2010) also reported clinical signs of swelling, pains and dry gangrene in humans after envenoming by *Echis ocellatus*. Pains have been a constant factor in all reported cases of viper bites, apparently due to cytotoxic effects of cytotoxic phospholipase A_2 (Michael *et al.,* 2013). L-amino acid oxidases, hyaluronidases and phosphodiesterases have all been implicated in pain and inflammation (Fox, 2013). Pains and inflammation could be responsible for the arching of the backs, depression and aggression shown by the mice. There is therefore some similarities in the clinical manifestation of *Echis ocellatus* envenoming between humans and Swiss albino rats. On post-mortem, the rats inoculated with the venoms of *Echis ocellatus* had severe haemoperitonium and haemothorax; the haemorrhagic syndrome is one of the most serious consequences of viperid snake envenomations (Gutierrez *et al.,* 2005). The pathogenesis of venom-induced haemorrhage involves direct damage to microvessels, performed by haemorrhagic toxins, combined with a wide variety of effects that viperid venoms exert in haemostasis (Gutierrez and Rucavado, 2000; Gutierrez *et al.,* 2005). Thus, capillary disruption and hemostatic disturbances act in harmony to provoke severe haemorrhage in viperid snakebites, although haemorrhagic toxins by themselves can induce bleeding in the absence of haemostatic alterations (Gutierrez *et al.,* 2005). Snake venom haemorrhagic toxins are zinc-dependent metalloproteinases which act mostly on the vascular endothelia thus leading to leakages of intact blood into the surrounding spaces. This is also responsible for the generalized congestion seen on most of the organs in *Echis ocellatus* envenomated rats.

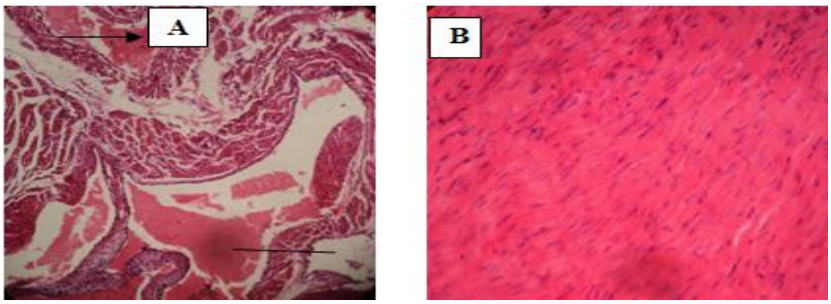

Fig. 1: Photomicrograph of heart section of rats.
A: Group experimentally envenomated with the venom of *Echis ocellatus.* Arrow showing necrosis, line showing congestions.
B: Control group showing a normal heart (H and E x400).

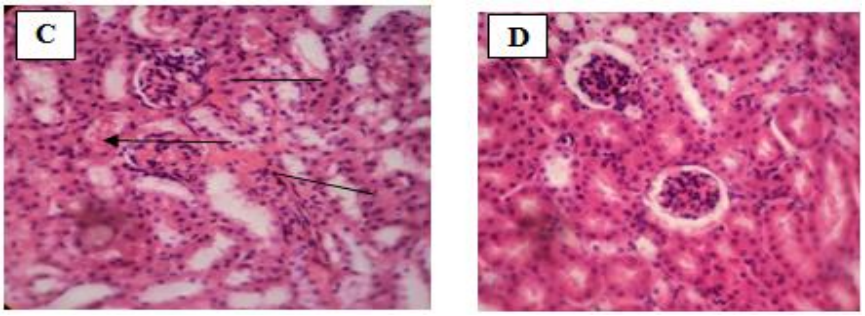

Fig. 2: Photomicrograph of kidney section of rats.
C: Group experimentally envenomated with the venom of *Echis ocellatus.* Arrow showing necrosis, line showing congestions.

B: Control group showing a normal kidney (H and E x400).

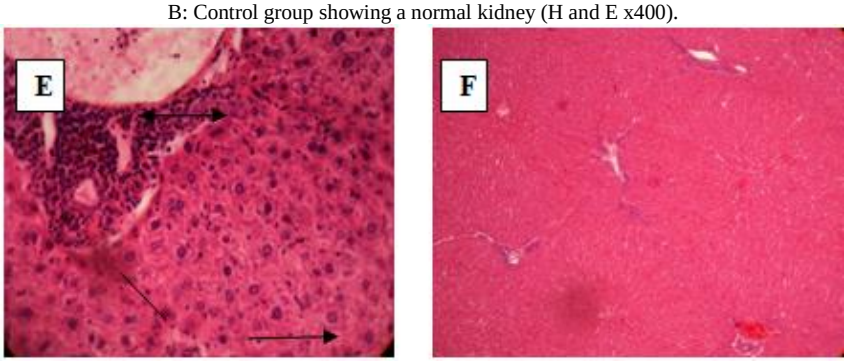

Fig. 3: Photomicrograph of liver section of rats.
E: Group experimentally envenomated with the venom of *Echis ocellatus.* Arrow showing necrosis, line showing congestions and double arrow showing perivascular cuffing.
B: Control group showing a normal liver (H and E x400).

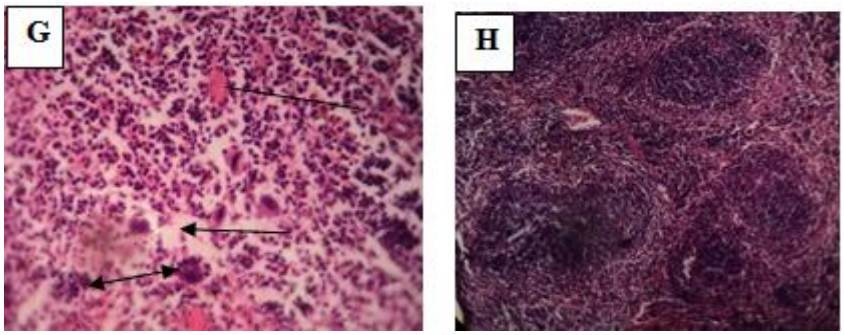

Fig. 4: Photomicrograph of spleen section of rats.
G: Group experimentally envenomated with the venom of *Echis ocellatus.* Arrow showing necrosis of lymphocytes, line showing congestions and double arrow showing presence of megakaryocytes.
H: Control group showing a normal spleen (H and E x200).

Snake venom myotoxins that specialize in rapid necrotization of skeletal muscle fibres contribute to prey immobilization as well as to its pre-digestion (Lomonte and Rangel, 2012). Like skeletal muscles, cardiac muscles are equally striated and vulnerable to snake venom myotoxins (Munawar *et al.*, 2014). Cardiac pathologies expressed by snake venoms in this study were severe cardiac haemorrhages and necrosis. All the rats inoculated with *Echis ocellatus* venom showed severe congestion and necrosis (Fig. 1). Although cardiac effects such as palpitations, arrhythmias, and hypotension are common clinical findings of *Naja* envenomations, cardiac damages are mostly seen at autopsy (Yusuf, 2019; Munawar *et al.*, 2014; Yusuf *et al.*, 2015). While the congestion can be related to the activities of snake venom zinc metalloproteinases (Gutierrez and Rucavado, 2000; Gutierrez *et al.*, 2005), the necrosis results from the activities of cardiotoxins (Munawar *et al.*, 2014), cytotoxins (Jamunaa *et al.*, 2012; Conlon *et al.*, 2013), and myotoxins (Angulo *et al.*, 2008; Lomonte and Rangel, 2012).

There is a broad spectrum of renal pathological changes involved in snake envenomation (Indraprasit *et al.*, 1986). Being a highly vascularized organ, the kidney is prone to venom toxicity (Pinho *et al.*, 2005; Visith, 2006). Clinical features suggestive of kidney involvement in snake envenomations include proteinuria, haematuria and acute renal failure (Visith 2006, Abubakar *et al.*, 2010a; WHO, 2010). Tubular necrosis is an important pathological counterpart of acute renal failure (Schneemann *et al.*, 2003; Polianna *et al.*, 2013; Amany *et al.*, 2014). Pathological changes induced by mice experimentally inoculated with the venom of *Echis ocellatus* in this study include congestion and focal areas of necrosis of the renal tubular epithelium (Fig. 2). Wagstag *et al.* (2008) attributed the haemorrhages to the activities of snake venom metalloproteinases on the intra-tubular capillaries. Myotoxins, cytotoxins and other non-classified snake venom protein were reported to be responsible for the necrosis (Koh *et al.*, 2006; Visith, 2006; Polianna *et al.*, 2013).

Snake venom has a wide range of biological activities (Al-Sadoon *et al.*, 2013). These affect a lot of body tissues and organs including the liver (Abdel-Moneim *et al.*, 2010; Abdel-Moneim *et al.*, 2013). Liver is considered as one of the targets for cobra venom

proteins (Abdel-Rahman *et al.,* 2013). Hepatic injury due to cobra envenoming was reported by Rahmy and Hemmaid (2000). Apart from both qualitative and quantitative changes in liver enzymes (Abdel-Rahman *et al.,* 2013; Amany *et al.,* 2014), there is paucity of information on hepatic damage induced by *Echis ocellatus* venom. In this study, rats inoculated with the venoms of *Echis ocellatus* showed histopathological lesions of congestion, necrosis of the hepatocytes, and perivascular cuffing (Fig. 3). Haemorrhages of vital body organs is attributed to the activities of haemorrhagins such as zinc metalloproteinases, L-amino acid oxidases and other toxic venom proteins (Gutierrez and Rucavado, 2000; Koh *et al.,* 2006; Amany *et al.,* 2014), while the necrosis can be attributed to cytotoxins present in *Echis ocellatus* venom (Koh *et al.,* 2006; Jamunaa *et al.,* 2012; Munawar *et al.,* 2014).

Table 1: Erythrocytic indices of the envenomated and control groups

Indices	Test Group	Control
Packed cells volume (%)	26 ± 0.7723	44 ± 0.5213
Haemoglobin (g/dl)	8.67 ± 0.4217	14.23 ± 0.3871
Erythrocytes (X 10^6/L)	± 0.5617	9.0 ± 0.3938
Total protein (g/dl)	3.2 ± 1.1623	8.6 ± 0.2928
Mean corpuscular volume (fl)	69.565	48.889
Mean corpuscular haemoglobin (pg)	21.675	15.556
Mean corpuscular haemoglobin concentration (g/l)	33.346	31.818

Table 2: Leukocytic indices of the envenomated and control groups

Indices	Test Group	Control
Leukocytes (X 10^9/l)	12.0±0.5429	12.12 ± 0.2419
Neutrophils (%)	9.0±1.2937	14.96 ± 0. 4527
Lymphocytes (%)	56±0.1923	67.24 ± 0.6123
Monocytes (%)	0.0±0.00	1.6 ± 0.2844
Eosinophils (%)	0.0±0.00	1.2 ± 0.5331
Basophils (%)	0.0±0.00	0.0±0.00
Band cells (%)	0.0±0.00	0.0±0.00

After inoculation with the venom of *Echis ocellatus*, the spleen which is a secondary lymphoid organ showed histopathological signs of necrotic lymphocytes resulting from the activities of these cells in fighting invading toxins (Fig. 4). Circulating immature thrombocytes (megakaryocytes) seen were probably due to bone marrow response as a result of massive destruction of circulating platelets and subsequent in coagulation of the blood, and severe congestion associated with decreased blood pressure and, thus decreased venous return to the heart leading to accumulation of blood in body organs (Fig. 4).

Haematological manifestation of snake envenomations has significant importance in the diagnosis and treatment of *Echis ocellatus* envenomation (Theakston *et al.,* 2003a; Gutierrez *et al.,* 2005; Abubakar *et al.,* 2010c; Warrell 2010; Habib, 2013; Yusuf *et al.,* 2015). Anaemia is a common feature of *Echis ocellatus* envenomation (Warrell, 1983; Furtado *et al.,* 2003; Lee, 2009; Gutierrez *et al.,* 2005; Koh *et al.,* 2006). In this study, anaemia was observed for the *Echis ocellatus* group (Table 1). The group inoculated with the venom of *Echis ocellatus* had severe anaemia, spherocytosis, anisocytosis, poikilocytosis, polychromatia, slight leukopenia, neutrophilia, monocytopenia, lymphopenia and low RBC count (Table 1 and Table 2), thereby agreeing with earlier reports of haemotoxicity by viper venom (Koh *et al.,* 2006, Theakston *et al.,* 2003b).

Conclusion: The median lethal dose (LD_{50}) of the venom of *Echis ocellatus* used in this study was 2.82 mg/kg by intraperitoneal route of administration. On experimental envenomation with *Echis ocellatus* venom, clinical signs of stretching, restlessness, aggression, shivering, rough hair coats and dry gangrene at the site of injection were observed. On post-mortem, severe haemopericardium and haemothorax were observed. *Echis ocellatus* venom caused congestion and myocardial necrosis in the hearts of experimentally envenomated rats. The venom caused congestion and focal areas of necrosis of the renal tubular epithelium in the kidney. In the liver, *Echis ocellatus* venom caused necrosis of hepatocytes and perivascular cuffing, with congested sinusoidal spaces. The spleen showed signs of congestion, necrosis of lymphocytes and presence of megakaryocytes. The blood picture indicated anaemia and a decrease in the population of circulating erythrocytes. There was no significant effect on the leukocytic series except for slight leukopenia resulting from lymphopenia and neutrophilia. Anisocytosis, poikilocytosis and polychromacia were also observed on the stained slides.

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