

***Cerastes cerastes gasperettii* venom Induced Hematological Alterations and Oxidative Stress in Male Mice**

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The desert horned vipers (*Cerastes cerastes gasperettii*; *C. c. gasperettii*) are the most familiar snakes of the great deserts of North Africa and the Middle East, including the Saudi Arabia. They are responsible for many human snake bites. The current study was designed to investigate the hematological effects and oxidative stress induction in lung, heart and spleen after *C. c. gasperettii* envenomation. Thirty six male Swiss albino mice were randomly divided into 2 groups, Control group injected intraperitoneally (i.p.) with saline or LD₅₀ dose envenomed group i.p. injected with venom at a dose of 978 µg/kg body weight (bwt). Mice were sacrificed after 1, 3 and 6 hrs from the injection. The number of white blood cells (WBC) was counted in envenomation and non-envenomation groups. Also, hemoglobin (Hb) was determined. In addition, lipid peroxidation (LPO), nitric oxide (NO), glutathione (GSH) levels and catalase (CAT) activity were measured in lung, cardiac and splenic homogenates. Statistical analyses were carried out using the unpaired student t test. The differential WBC count showed difference between envenomated and non-envenomated mice, which was mainly attributable to increase in neutrophils, monocytes eosinophils, and basophils in the envenomation mice ($p < 0.001$). In the envenomation mice, the amounts of Hb were significantly lower compared to those of the non-envenomation group ($p < 0.001$). In addition to the hematological alterations, *C. c. gasperettii* envenoming was associated to significant increasing in oxidative stress levels. Moreover, congestion of the alveolar capillaries in lung, inflammatory cell infiltration and myonecrosis in heart and splenomegaly were observed after 6 hrs of envenomation. Based on these observations, we may conclude that the LD₅₀ of *C. c. gasperettii* venom causes hematological alterations in mice, characterized by elevated oxidative stress levels and histological alterations in heart, lung and spleen tissues.

Keywords: *Cerastes cerastes gasperettii*; Blood elements; Oxidative stress; Mice.

Following a snakebites of family Viperidae, various local and systemic alterations occur such as haemorrhage, bleeding disorders, edema and myonecrosis leading to tissue loss or organ dysfunction (Meier and White, 1995). Usually these effects develop very rapidly after

the snakebites, making neutralization by anti-venoms very difficult, especially if serotherapy is delayed due to either late access to medical care or scarcity of anti-venoms.

Vipers are widely distributed throughout Africa and Eurasia. *Cerastes cerastes* mainly inhabits the North Africa, and also Asia including Saudi Arabia. According to the records of the Ministry of Health, the incidence of snakebites in

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Saudi Arabia is approximately 2,494 cases per year (Al-Asmari and Abdo, 2006).

Viperidae venoms are rich sources of hydrolytic enzymes that produce a complex pattern of clinical and toxic effects (Hamza *et al.*, 2010a; Hamza *et al.*, 2010b). The action of venom is the combined effect of all components present in the venoms (Cherifi and Laraba-Djebari, 2013). However, most venom components produce beneficial effects when they act alone (Cherifi *et al.*, 2010). Snake venom also contains non-protein components including citrate, metal ions, carbohydrates, nucleotides as well as low concentrations of free amino acids and lipids (Mion *et al.*, 2002).

Hematological changes in erythrocytes, leukocytes, platelets, coagulation factors, and fibrinogen are some toxic effects caused by animal venoms in humans (Netto *et al.*, 2004; Thomazini *et al.*, 1991). In envenomated victims, liver and erythrocytes are always threatened with subsequent generation of reactive oxygen species (ROS), which may induce capillary perfusion resulting in cell lysis and local tissue damage (Sebastin Santhosh *et al.*, 2013). In addition, ROS are involved in the inflammatory reactions, thereby affecting the cellular physiology and play a significant role in the pathological conditions (Carroll *et al.*, 2007). The free radicals, apart from being involved in damaging cellular components, play a significant role in venom induced toxicity.

Haemorrhage, edema, myonecrosis and other tissue alterations follow serious viperian strike which invariably gives rise to massive accumulation of free radicals which contributes to the venom toxicity. This study therefore was designed to investigate the hematologic effects and oxidative stress inductions of *C. c. gasperettii* envenomation.

MATERIALS AND METHODS

Chemicals

Tris-HCl was purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid and trichloroacetic acid were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent.

Snake collection

The crude venom was obtained by milking of about 20 specimens of *C. c. gasperettii* snakes collected from the central region of Saudi Arabia. The approximate LD₅₀ (978 µg/kg body weight) of the venom was calculated in our previous study (Al-Sadoon *et al.*, 2013) and followed the method described by Meier and Theakston (1986).

Experimental animals

Experiments were performed on male Swiss albino mice, 6–8 weeks old, weighing 22 ± 5 g. The animals were obtained from Schistosome Biological Supply Center at Theodor Bilharz Research Institute, Giza, Egypt. Animals were kept in wire bottomed cages, in a room under standard condition of illumination with a 12 h light-dark cycle at 25 ± 1 °C. They were provided with tap water and balanced diet *ad libitum*. They were acclimatized to the environment for one week prior to experimental use. We have followed the European Community Directive (86/609/EEC) and national rules on animal care that was carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals 8th edition.

Experimental groups

To study the effect of *C. c. gasperettii* venom, thirty six male Swiss albino mice were randomly divided into:

Control group (n=18): mice were injected intrapretoneally (i.p.) with 0.2 ml saline solution/mouse, and sacrificed after 1, 3 and 6 hrs from injection.

The LD₅₀ dose envenomed group (n=18): mice were i.p. injected with 0.2 ml saline solution containing venom at a dose of 978 µg/kg body weight (bwt), and mice were sacrificed after 1, 3 and 6 hrs from the injection.

A few pieces of lung, heart and spleen were fixed with 10% neutral buffered formalin for histopathological investigations, whereas the majority of pieces were homogenized in ice-cold medium containing 50 mM Tris-HCl, pH 7.4, and finally stored at -20 °C until use in the various biochemical determinations.

Differential white blood cell counts

Differential white blood cell counts were determined using standard blood smears as described by Koepke (1991).

Biochemical studies

Hemoglobin content

The blood sample was transferred to a

clean glass tube containing heparin (sodium salt, 20 µl/ml of blood) as anticoagulant and then used to determine hemoglobin. Hemoglobin was measured by mixing 20µl of blood with 2.5 ml of Drabkin solution. Cyanmethemoglobin formation was determined by absorbance at 540 nm and compared to that of a hemoglobin standard (Saunders, 1998).

Oxidative stress

Homogenates of lung, heart and spleen were used to determine lipid peroxidation (LPO) by reaction of thiobarbituric acid (TBA) (Ohkawa *et al.*, 1979). Similarly, those homogenates were used to determine nitrite/nitrate (nitric oxide; NO) (Green *et al.*, 1982) and glutathione (Ellman, 1959). Whereas, catalase (CAT) activity was measured according to Aebi (1984). The levels of oxidative stress parameters were expressed per mg protein, where protein in the different homogenates were determined according to Lowry *et al.* (1951).

Histopathological studies

Tissue samples were fixed in 10% neutral formalin for 24 hours and paraffin blocks were obtained and routinely processed for light microscopy. Slices of 4-5 µm were obtained from the prepared blocks and stained with hematoxylin-eosin. The preparations obtained were visualized using a Nikon microscope at a magnification of 400×.

Statistical analysis

The data are presented as means ± SEM. Data were analyzed in SPSS version 17.0. Unpaired student's t-test was used a test of significance. $p < 0.05$ (a), $p < 0.01$ (*) and $p < 0.001$ (**) were considered to be significant.

RESULTS

White blood cells (WBC) count showed significant increase in *C. c. gasperettii* venom treated group after 1 and 3 hours (Table 1). However, WBC count was tending to reduce significantly after 6 hours of venom injection. As shown in the Table 1, there were significant increase ($p < 0.001$) in the percentage of neutrophils, monocytes, eosinophils and basophils at all the time intervals as compared to the control mice. However, There was a significant decrease ($p < 0.001$) in the percentage of lymphocyte counts in *C. c. gasperettii* venom treated group when compared to the control animals.

The concentration of haemoglobin reduced in all the time intervals of all mice received venom. In the mice which received venom dissolved in saline at a doses of 978 µg/kg bwt, the decreases in Hb were from 14.91 g/dL in the control to 12.32 g/dL after 1 hr and 13.97 g/dL after 3 hrs, Figure 1. The maximum decrease in haemoglobin occurred in the group receiving venom for 6 hrs (8.69 g/dL).

The results clearly demonstrated that, a single injection of *C. c. gasperettii* venom resulted in the elevated levels of oxidative stress parameters, Figure 2. These were evidenced by significant increase in the levels of both lipid peroxidation and nitric oxide in time dependent manner. However, after 3 and 6 hrs of venom injection, NO levels were significantly decreased in heart homogenate. Also, the glutathione (GSH) content was significantly reduced in the examined tissues of

Table 1. White blood cell and differential white blood cell counts in mice treated with *C. c. gasperettii* venom

Group	Control	1 hour envenomation	3 hours envenomation	6 hours envenomation
WBC ($10^3/\text{mm}^3$)	3.7±0.2	5.5±0.3 ^{a**}	6.3±0.3 ^{a**}	2.5±0.4 ^{a**}
Neutrophils (percentage)	23.3±1.5	43.5±2.8 ^{a**}	42.7±2.8 ^{a**}	55.5±3.7 ^{a**}
Lymphocytes (percentage)	75±2.8	55.5±3.3 ^{a**}	55.3±3.7 ^{a**}	41.5±2.6 ^{a**}
Basophils (percentage)	0.0±0.0	0.0±0.0	0.7±0.2 ^a	1.5±0.3 ^a
Monocytes (percentage)	0.7±0.1	1.0±0.2 ^{a*}	1.0±0.1 ^{a*}	1.7±0.2 ^{a*}
Eosinophils (percentage)	0.0±0.0	0.0±0.0	0.7±0.2 ^{a**}	0.5±0.1 ^{a**}

Values are means ± SEM. a: statistically significant differences with respect to vehicle control group at <0.05 , * statistically significant differences at <0.01 and ** statistically significant differences at <0.001 , n=6.

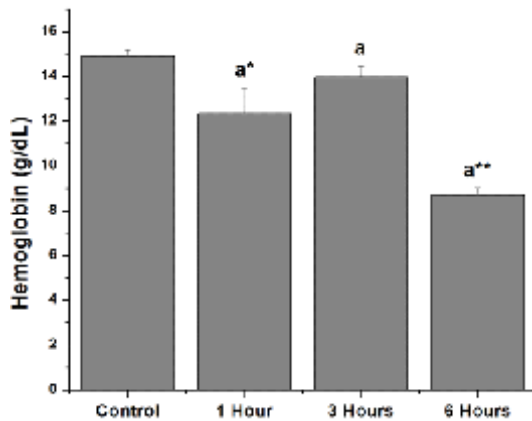


Fig. 1. Effect of *C. c. gasperettii* venom injection on hemoglobin level. Values are means \pm SEM. a: statistically significant differences with respect to vehicle control group at ≤ 0.05 , * statistically significant differences at ≤ 0.01 and ** statistically significant differences at ≤ 0.001 n=6

mice receiving the venom. Moreover, there was an elevation in CAT activity of heart in the envenomated mice (Figure 2).

Macroscopically, extensive diffuse hemorrhagic areas were found in all lung lobes of the animals in experimental injected groups. The lung tissue histological analysis from envenomated animals showed moderate alveolar capillary congestion and large quantities of erythrocytes diffusely distributed in the interstitial and intra-alveolar spaces (Figure 3b-d). Such hemorrhagic indication in the lung demonstrates the direct action of the venom. Histological analysis of the heart tissue from experimental animals revealed inflammatory cell infiltration and myonecrosis (Figure 4b-d), while in spleen analysis, there were increasing spaces between the cells revealing splenomegaly in time dependent manner (Figure 5b-d). Splenomegaly was evidenced by significant

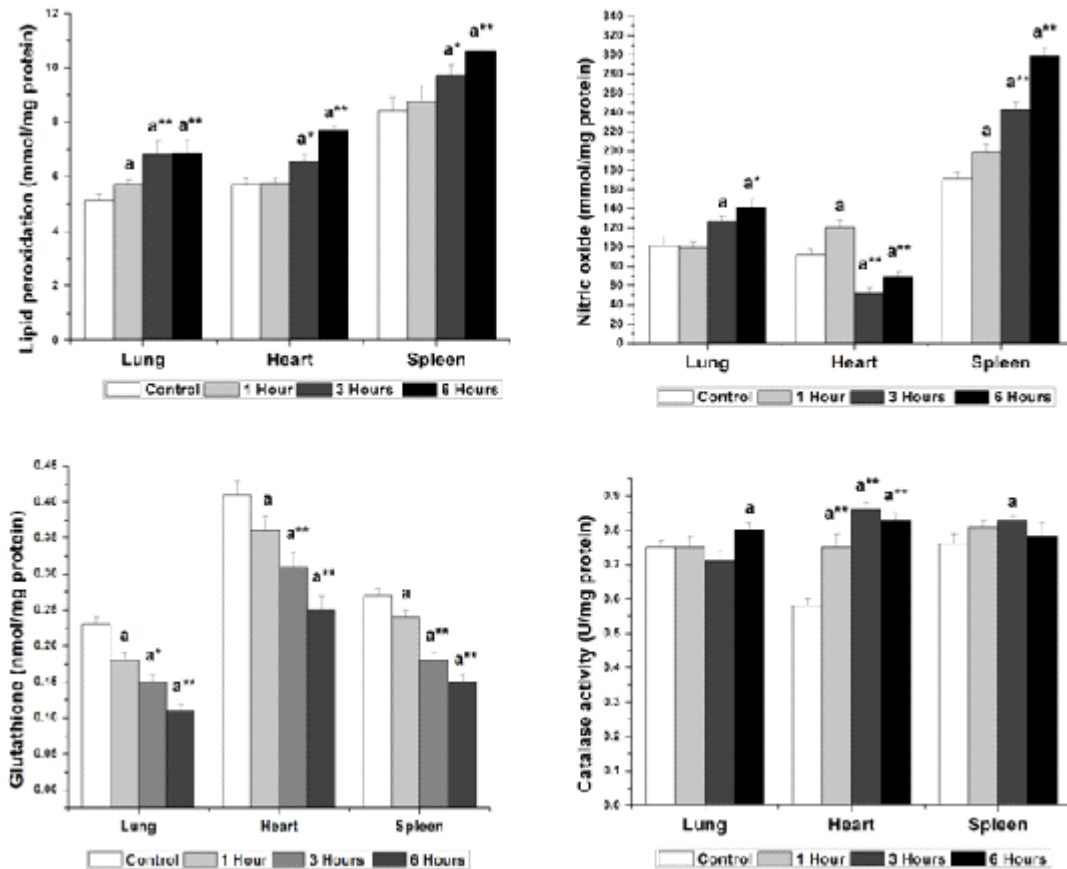


Fig. 2. Effect of *C. c. gasperettii* venom injection on lipid peroxidation, nitric oxide and glutathione levels and catalase activity. Values are means \pm SEM. a: Significant against vehicle control group at ≤ 0.05 , * significant at $p < 0.01$ and ** significant at ≤ 0.001 , n=6.

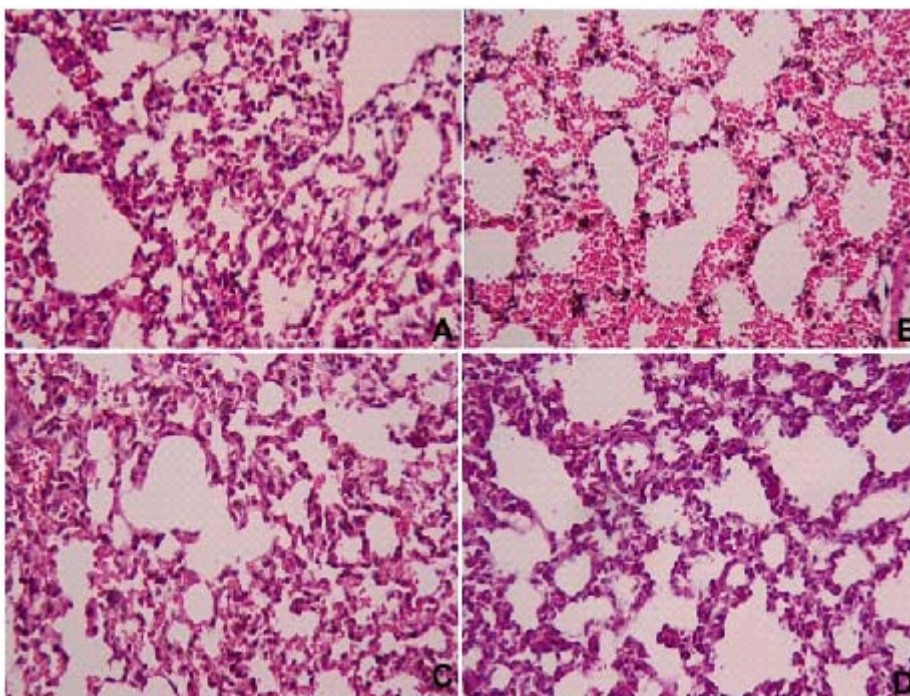


Fig. 3. Sections of lungs of the animals in different groups. a) Control lung with normal structure, b, c) lung of mice 1 and 3 hours after *C. c. gasperettii* venom injection, respectively, revealing presence of erythrocytes in the parenchyma, and d) lung of mice 6 hours after *C. c. gasperettii* venom injection showed extensive hemorrhagic areas revealing capillary congestion and presence of erythrocytes in the parenchyma. (H & E, 400x).

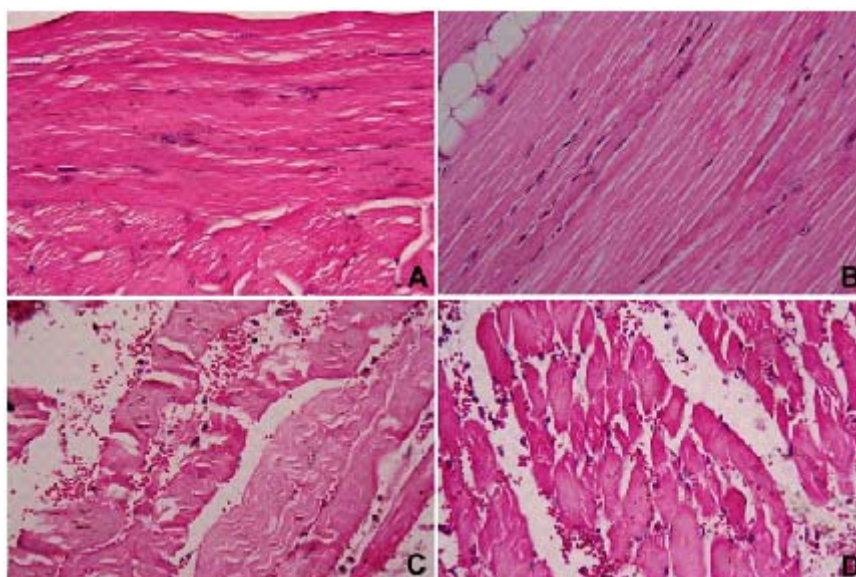


Fig. 4. Sections of hearts of the animals in different groups. a) Longitudinal section of cardiac muscle from mice in control group with normal structure, b) haemorrhage and rare mixed character inflammatory cell infiltration in the longitudinal section of cardiac muscle 1 hour after *C. c. gasperettii* venom injection, c) mild mixed character inflammatory cell infiltration and myonecrosis in the transversal section of mice injected with the venom after 3 hours, and d) intense mixed character inflammatory cell infiltration and myonecrosis in the longitudinal section of cardiac muscle of mice injected with *C. c. gasperettii* venom after 6 hours. (H & E, 400x).

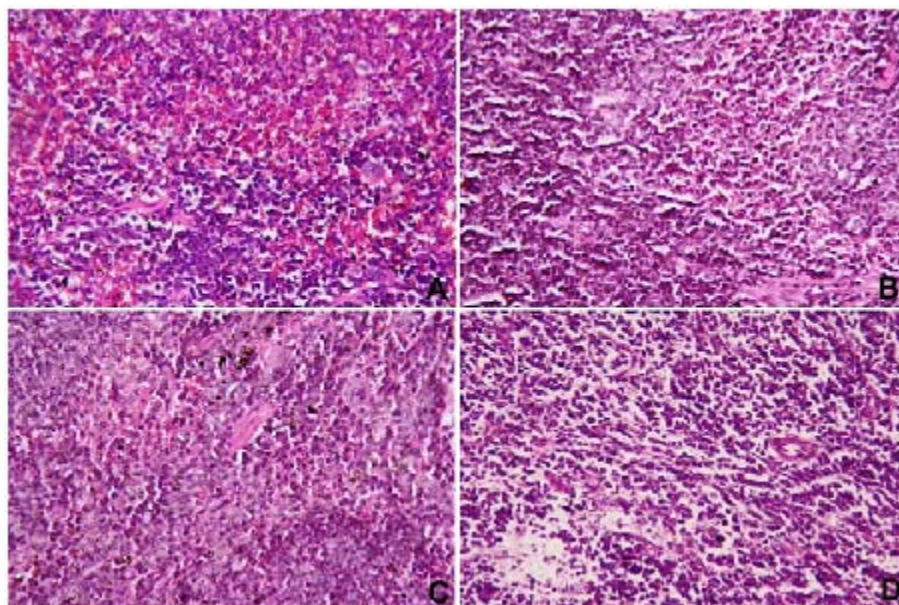


Fig. 5. Sections of spleens of the animals in different groups. a) Control spleen with normal structure, b, c and d) spleen of mice 1, 3 and 6 hours after *C. c. gasperettii* venom injection, respectively, revealing increase space between the cells indicating the splenomegaly. The increase in space between the cells

increase in spleen weight (data not shown). In addition to the splenomegaly, numerous small islands of darkly stained cells in red pulp of subcapsular area and numerous aggregations of megakaryocytes were present.

DISCUSSION

It is well established that some venom components have beneficial effects when acting in isolation. *C. c. gasperettii* venom is a mixture of protein components with multiple actions including coagulation (Bazaa *et al.*, 2005; Cherifi and Laraba-Djebari, 2013; Laraba-Djebari *et al.*, 1995). These proteins may induce hemorrhage and capillary permeability disorders, through their disintegrin domain or related proteins that disrupt primary hemostasis by acting on platelet adhesion. Thus, a single molecule can be endowed with several activities. The structural differences between proteins, natural factors of hemostasis, as well as the multiplicity of target components of the same venom, are elements that could explain the efficiency of partial immunotherapy (Cherifi and Laraba-Djebari, 2013).

Several molecules from *C. c. gasperettii* venom act on hemostasis, such as RP34, a serine

proteinase which consists of two subunits of 48.5 kDa (Cherifi and Laraba-Djebari, 2013). Another serine proteinase, a thrombin-like molecule denominated afaâcytin, was purified and characterized (Laraba-Djebari *et al.*, 1995). Afaâcytin presents caseinolytic, arginine-esterase and amidase activities. It is a homodimeric proteinase with two subunits, alpha and beta, which have the same apparent molecular mass (40.0 kDa for each unit) and are indistinguishable in the absence of reduction or/and deglycosylation (Laraba-Djebari *et al.*, 1995). Both \pm and 2 chains are N-glycosylated. The two chains present the same N-terminal sequence (20 residues) which is similar to the sequence of other proteinases isolated from snake venom. Fibrinogenases (serine proteinases or metalloproteinases) are widespread in Viperidae venoms. They hydrolyze fibrinogen and/or degrade the fibrin clot, enhancing the effect of hemorrhagic metalloproteinases that give rise to pathological bleeding.

Reasons of leucocytosis could be nephritis and nephrotoxicity. Experimental animals exposed to envenomation, including snake biting could be classified as a type of exposure to stress, which in turn leads to disruption in the production

of white blood cells, and has an impact on all of the different blood components that was also mentioned by Al-Sadoon and Fahim (2012). Leucopenia that observed after 6 hours of venom injection may be due to bone marrow failure as infection, tumor, or abnormal scarring, collagen-vascular diseases, disease of the liver or spleen while leucocytosis may be due to anemia, infectious diseases, inflammatory disease, leukemia, physical stress, tissue damage (Al-Sadoon and Fahim, 2012).

Mackay et al. (1969) reported that cobra venom from the elapid family cause drop in the number of white blood cells a short period after injection. This is consistent with the results of the present study. Leucopenia may be due to liver failure (Kumar and Clark, 2005) which was observed as a result of envenomation. In the present study, the count of WBC increased after 1 and 3 hours and this observation is consistent with observations of Amin *et al.* (2008) who reported that Bangladesh snake venom caused leucocytosis after a period of injection. Al-Sadoon *et al.* (2012) hypothesized that a sympathetic effect, as a result of the stress experienced by the victims, could release temporarily WBCs from the marginal pools.

Snake bites are most often accompanied by signs of inflammation and local tissue damage (Nelson, 1989). Neutrophils and macrophages are induced to produce superoxide radical anion which belongs to a group of reactive oxygen species (ROS) and this reacts with cellular lipids leading to the formation of lipid peroxides and the observed necrosis (Valko *et al.*, 2007). As the origin of oxidative stress is the mitochondrial respiratory electron transport chains (Fletcher *et al.*, 1991), it is possible that mitochondrial death mediates venom-induced cellular damage (Haffor and Al-Sadoon, 2008).

High levels of lipid peroxidation and nitric oxide and activity of catalase are indicatives of an oxidant condition. Oxidative stress and alteration of protein contents suggesting membrane destruction, enzyme release, and protein loss due to venom-direct tissue damage (Silva *et al.*, 2011). Oxidative stress may occur when the balance that exists between the formation of ROS and their removal by endogenous antioxidant scavenging compounds (Abdel-Moneim *et al.*, 2011) is disrupted by excessive production of ROS,

including $^{\cdot}\text{O}_2$, H_2O_2 , and $^{\cdot}\text{OH}$. Alternatively, oxidative stress may also be caused by inadequate antioxidant defenses due to changes in superoxide dismutase and CAT activities and glutathione level (Gutteridge, 1995). The induction of CAT enzyme after the venom inoculation was probably necessary for host defense. Furthermore, oxidative stress is related to rhabdomyolysis, a clinical complication in snakebites (Silva *et al.*, 2011).

Lipid peroxidation is one of the primary manifestations of oxidative damage initiated by ROS and is considered to be one of the suggested cytotoxic mechanisms of snake venom. Venom-induced oxidative stress and malfunctioning of vital organs through membrane destruction, protein loss and enzyme release have reported with few studies (Santhosh *et al.*, 2013). A significant increase in lipid peroxidation was observed at different time points of venom injection in different organs of venom-treated animals. In this situation, myoglobin degradation results in the release of free iron that catalyses ROS production, initiating lipid peroxidation and increasing the redox imbalance in different organs (Othman *et al.*, 2014). Such oxidative damage can lead to cellular and/or tissue alterations that compromise the organ function and cause dysfunction in vital organs.

Many studies demonstrated the involvement of pro-inflammatory substances in venom-induced oxidative stress damages (Santhosh *et al.*, 2013). The current results clearly suggested that *C. c. gasperettii* venom stimulates NO production. These results agree with earlier study showing augmented level of NO in snakebite mice injected with *Naja haje* venom (Tohamy *et al.*, 2014).

The oxidative stress can be neutralized through non-enzymatic mechanisms that comprise the cellular defense system. The alternation in glutathione level was investigated. The *C. c. gasperettii* venom drastically depleted the GSH contents in the tested organs. The induction of the non-enzymatic defensive mechanism on exposure to *C. c. gasperettii* venom could be an adaptive or a compensatory response that enables the cells to overcome the damage (Santhosh *et al.*, 2013). Therefore, a generalized oxidant condition seems to be present and can contribute to the *C. c. gasperettii* venom toxicity.

In the present study, *C. c. gasperettii*

venom induced moderate to severe histopathological changes in vital organs. In the present studies, lung sections showed moderate alveolar capillary congestion and infiltration of erythrocytes and lymphocyte within bronchial alveoli and dilation of bronchi. The precise mechanisms responsible for these changes have not been completely clarified, however, metalloproteinases may play a role in release of pro-inflammatory cytokines. The haemorrhagic metalloproteinases of *Viperidae* snake venom are also considered to be responsible for local haemorrhage, degrading components of the vascular basement membrane, followed by total disintegration of the vascular structure (Fox and Serrano, 2005; Silveira *et al.*, 2004). A similar mechanism is suggested as responsible for the systemic bleeding (Kawano *et al.*, 2002). Also, phospholipase A₂ (PLA₂) induced local myonecrosis and lymphatic vessel damage (Gutierrez *et al.*, 2009; Gutierrez and Ownby, 2003).

Cardiac injury is one of the well-known toxicities related to snakebites. Cardiotoxicity is due to the presence of myotoxic PLA₂ and other cardiotoxin(s) that are responsible for cellular necrosis and cytotoxicity (Asad *et al.*, 2014). Our results revealed that *C. c. gasperettii* venom caused inflammatory cell infiltration and myonecrosis. Similarly, venom of *Vipera apis*, on the other hand, causes both haemorrhage and myonecrosis in muscular tissue. Venom of *Vipera russelli siamensis* causes myonecrosis despite the fact that it does not cause haemorrhage in muscular tissues (Tu *et al.*, 1969). It was established that the venoms of *Bitis sp.* cause haemorrhage but not myonecrosis in muscular tissue (Homma and Tu, 1971).

We have revealed that the histostructure of the spleen is disturbed under the influence of *C. c. gasperettii* venom. Presence of numerous small islands of darkly stained erythropoietic precursor cells seen in enlarged spleens of mice indicates extramedullary erythropoiesis in spleen. Presence of numerous aggregations of megakaryocytes in red pulp also indicates production of platelets in spleen. The above observation indicates a response by spleen to such haematological changes by hyperplasia and extramedullary haemopoiesis (Silva *et al.*, 2012).

Finally, our data have shown that *C. c. gasperettii* venom exert significant effects on the lung, heart and spleen. These effects concern mainly hematologic alterations and oxidative stress. The action of the venom is probably the result of the action of several components. From a complex mixture, one can expect synergisms and even antagonisms. Further studies with purified components of *C. c. gasperettii* venom are necessary to obtain detailed information about its mechanisms of action and its potential biotechnology application. The present data are, therefore, more relevant for the clinical view of the *C. c. gasperettii* intoxication.

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