Histochemical and Biochemical effects induced by LD₅₀ of Cerastes cerastes gasperetti crude venom in mice

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Abstract: Snake venoms comprise mixtures of enzymes and proteins that act on vital systems of the victim. The present study aimed to investigate the histochemical and biochemical effects of the horned viper (Cerastes cerastes gasperetti: Ccg) snake venom in mice. Thirty six male adult Swiss albino mice (20–25g) were injected intraperitoneally and divided into 2 groups (n=18): control group injected with 200 µl saline solution and group (2) injected with LD₅₀ of Ccg venom (0.978 mg/kg). Liver, kidney and testis were collected from six mice after 1, 3 and 6 hours, post envenomation. Histological examination of testis sections after 1, 3 and 6 hours, post envenomation showed seminiferous tubules displaying an impairment of spermatogenesis stages arrangement with severe vacuolization of most affected tubules and appearance of pyknotic spermatocytes. Histochemical investigations revealed marked reduction in both carbohydrates and proteins after injection of LD₅₀ of Ccg crude venom at the different time. Oxidative stress biomarkers malondialdehyde (MDA) and nitrite/nitrate levels, antioxidants glutathione (GSH) content and catalase (CAT) activity were assayed in tissues homogenates. The venom induced significant increases in the levels of MDA and nitrite/nitrate while the content of GSH and the activity of CAT were significantly decreased, especially after 6 hours of envenomation. The results revealed that the Ccg venom induced time-dependent significant increases in the levels of MDA and nitrite/nitrate in all examined organs, especially in the hepatic tissues. It can be concluded that cytotoxic effects of Ccg venom might be related to induction of excessive oxidative stress and histochemical alterations in organs of the envenomated animals.


Keywords: Cerastes cerastes gasperetti, histochemical, oxidative stress, liver, kidney, testis.

1. Introduction

Snake venom is a complex mixture of many substances, such as toxins, enzymes, growth factors, activators, and inhibitors with a wide spectrum of biological activities (Theakston, 1983; Rahmy and Hemmaid, 2000). They are also known to cause different metabolic disorders by altering the cellular inclusions and enzymatic activities of different organs (Aiesenbarg, 1981).

The Kingdom of Saudi Arabia and surrounding areas are inhabited by several venomous snakes of medical importance. Among them are the families Elapidae, Hydrophidiae, Viperidae and Atracaspidae, in addition to the innocuous family Colubridae, which includes weakly venomous, back-fanged representatives (Gasperetti, 1977). From the medical point of view, vipers, with their highly sophisticated venom delivery system, are the most important snakes in Saudi Arabia since they are responsible for most of the snakebites affecting humans, and the sand viper (Cerastes cerastes gasperetti; Ccg ) is the most frequent of all (Gasperetti, 1988). The horned viper Ccg is widely distributed from Africa to South-Western Asia. It is the most common snake in Saudi Arabia especially in the central region (Al- Sadoon, 1989), and it poses a serious medical problem (Djebari and Martin-Eauville, 1990). Envenomning by the Cerastes cerastes viper is quite common both in the area of its occurrence and among snake-keepers because of the high popularity and availability of this species (Valenta et al., 2010). The clinical course of intoxication is usually not severe, presenting with mild discomfort, elevated temperature, nausea, sometimes vertigo, vomiting accompanied by laboratory evidence of coagulation disorder without major clinical presentation, and minor local lesion. Some afflicted persons do not even seek medical consultation. For these reasons, publications on envenomning by the Cerastes viper are quite rare and mostly depict a typical course with coagulation disorder and renal failure (Lifshitz et al., 1995, 2000, 2002; Schneemann et al., 2004). In view of the paucity of information on the effects of Ccg crude venom, the present study was planned to investigate the effects of the snake crude venom on hepatic, renal and testicular tissues histochemical and biochemical parameters of mice.
2. Material and Methods

2.1. Cerastes cerastes gaspartii (Ccg) venom:

Cerastes cerastes gaspartii snakes were collected from the central region of Saudi Arabia. The snakes were kept in a serpentarium in the Zoology Department, College of Science, King Saud University. The snakes were warmed daily using a 100-watt lamp for nine hours, and water was always available. The snakes were fed purpose-bred mice every 10 to 14 days. The venom was milked from adult snakes, lyophilized and reconstituted in 1X phosphate- buffered saline prior to use. The approximate median lethal dose (LD\textsubscript{50}) of the crude venom was found to be 0.978 mg/ kg mice (Al – Sadoon et al., 2013).

2.2. Experimental design:

Thirty six adult male Swiss albino mice weighing 20-25 g were used. Mice were selected from Schistosome Biological Supply Center at Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. Animals were housed in standard condition and fed with normal diet and water ad libitum. Mice were divided into four groups as the following:

Group I: 18 animals were injected interaperitoneally (i.p) with 200 μL physiological saline (0.9 % NaCl) and served as a control.

Group II: 18 animals were received a single dose (0.978 mg/kg body weight) of the viper Cerastes cerastes gasperetti crude venom in 200 μL saline solution interaperitoneally (i.p).

Six animals of each group (I and II) were sacrificed at 1, 3 and 6 hours respectively post-injection of crude venom.

2.3. Histological and Histochemical Preparations:

The livers, kidneys and testes from the control and envenomated groups were rapidly excised after the previously mentioned duration, cut into small pieces and dropped in 10% neutral buffer formalin in which they were kept for appropriate time. After fixation, they were subjected to the normal procedure for paraffin embedding. Sections of testes were cut at the thickness of 5 microns and stained with haematoxyline-eosin (Drury and Wallington, 1981). Periodic Acid Schiff technique (PAS) (Hotchkiss, 1948), was used for the studying of general carbohydrate and Mercuric Bromophenol Blue technique (MBB) was used for the demonstrating of total proteins (Mazia et al.,1953) in livers, kidneys and testes. The preparations obtained were visualized using a light microscopy at a magnification of 400X.

2.4. Biochemical studies:

Pieces of liver, kidney and testis were homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl, pH 7.4. The homogenates were cold centrifuged at 500 xg for 10 min. The supernatant (10%) was used for the various biochemical determinations.

2.4.1. Determination of lipid peroxidation and nitrite/nitrate

Lipid peroxidation (LPO) was assayed colorimetrically in liver, kidney and testis homogenates according to the method described by Ohkawa et al. (1979). LPO determined by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% and were then heated in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) formed. The assay of nitrite/nitrate, as an indirect measure of NO production, was done according to the method described by Green et al. (1982) in liver, kidney and testis homogenates. In an acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide was coupled with N-(1-naphthyl) ethylenediamine. The resulting azo-dye had a bright reddish-purple color which could be measured spectrophotometrically at 540 nm.

2.4.2. Estimation of glutathione

The hepatic, renal and testicular glutathione (GSH) levels were determined according to Ellman (1959). The method based on the reduction of Elman's reagent (5, 5' dithiobis (2-nitrobenzoic acid) "DTNB") with GSH to produce a yellow compound. The reduced chromogen was directly proportional to GSH concentration and its absorbance was measured at 405 nm.

2.4.3. Estimation of catalase activity

According to the method of Aebi (1984), catalase (CAT) reacts with a known quantity of H\textsubscript{2}O\textsubscript{2}. The reaction is stopped after exactly 1 min with catalase inhibitor. In the presence of peroxidase (HRP), the remaining H\textsubscript{2}O\textsubscript{2} reacts with 3, 5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4- aminophenazon (AAP) to form a chromophore with color intensity inversely proportional to the activity of CAT in the original sample.

2.5. Statistical analysis

The data are presented as means ± standard error of the mean (SEM) and statistically analyzed using ANOVA test (version 17.00). Significance was set at the level of P ≤ 0.001 against control.

\[
\%\text{Difference} = \frac{\text{Mean of treated} - \text{Mean of control}}{\text{Mean of control}} \times 10
\]

3. Results

3.1. Histological observations:

Control testis section (Figure 1a) showed several regular seminiferous tubules surrounded by well defined basal lamina. The tubules are separated by
scanty connective tissue that contains blood capillaries and clusters of interstitial cells. The seminiferous tubules are lined with a complex stratified epithelium of spermatogenic cells in various stages of development. While testis sections of mice injected with LD₅₀ of Ccg venom for 1h, 3h and 6h showed seminiferous tubules displaying an impairment of spermatogenesis stages arrangement with severe vacuolization of most affected tubules and appearance of pyknotic spermatocytes (Figures 1b, c and d). Complete degeneration was noted in seminiferous tubules after 6 hours of Ccg injection.

![Figure 1](image1.png)

**Figure (1):** (a) Testis of mice in control group, showing the normal histological structure of the seminiferous tubules with different stages of spermatogenic layers and spermatozoa. (b) Testis of mice after 1 hour post Ccg envenomation, showing degenerated spermatocytes exfoliated to the lumen (arrows). (c) Testis of mice at the 3rd hour post Ccg envenomation, showing vacuolation in the seminiferous epithelium (arrows). (d) Testis of mice after 6 hours post Ccg envenomation, showing pyknotic spermatocytes (arrows) and complete degeneration. Sections were stained with HE, x400.

3.2. Histochemical observations:
3.2.1. General carbohydrates:
A considerable amount of carbohydrates in the cytoplasm of liver cells of control mice was noticed by PAS-technique, which gave a red or magenta colour (Figure 2a). The nuclei, however, appeared entirely PAS-negative staining, indicating absolute lack of carbohydrates. Injected mice with LD₅₀ of Ccg venom for 1 hour caused a decrease of total carbohydrates in the liver cells (Figures 2 b). A marked reduction of total carbohydrates was observed after 3 and 6 hours of LD₅₀ Ccg injection (Figure 2c and d).

![Figure 2](image2.png)

**Figure (2):** Sections of liver stained with PAS. a) Control mice, b) Ccg venom injected mice after 1 hour, c) Ccg venom injected mice after 3 hours and d) Ccg venom injected mice after 6 hours (x400).

The histochemical examination of kidney sections of control group mice stained with PAS technique showed the presence of polysaccharides in the form of PAS positive materials in the parietal and visceral walls of the Bowman’s capsule, capillaries of the glomeruli, basement membrane of the proximal and distal convoluted tubules and the brush border of the proximal convoluted tubules (Figure 3a). Light microscopy of the kidney sections of injected mice with LD₅₀ of Ccg venom post 1 and 3 hours showed a decrease in the PAS positive material in the mesangial cell and matrix of the glomeruli (Figures 3 b and c). A marked reduction of total carbohydrates was observed after 6 hours of LD₅₀ Ccg injection (Figure 3d).

![Figure 3](image3.png)

**Figure 3:** Sections of kidney stained with PAS. a) Control mice, b) Ccg venom injected mice after 1 hour, c) Ccg venom injected mice after 3 hours and d) Ccg venom injected mice after 6 hours (x400).

Although, a considerable amount of carbohydrates in the cytoplasm of testis tissue of control mice was noticed (Figure 4a). The injection of mice with LD₅₀ of Ccg venom for 1 and 3 hours caused a decrease of total carbohydrates in the testis
tissue (Figures 4 b and c). Moreover, a marked reduction of total carbohydrates was noticed after 6 hours (Figure 4d).

**Figure 4:** Sections of testis stained with PAS. a) Control mice, b) *Ccg* venom injected mice after 1 hour, c) *Ccg* venom injected mice after 3 hours and d) *Ccg* venom injected mice after 6 hours (x400).

### 3.2.2. Total proteins:

Total proteins were demonstrated in liver cells of control mice as deeply blue stained with MBB Stain (Figure 5a). Injection mice with LD₅₀ of *Ccg* venom for 1 and 3 hours induced a slight decrease in the protein content of the liver cells (Figures 5 b and c). More reduction in proteins was manifested in the cells after 6 hours of envenomation (Figure 5 d).

**Figure 5:** Sections of liver stained with bromophenol blue. a) Control mice, b) *Ccg* venom injected mice after 1 hour, c) *Ccg* venom injected mice after 3 hours and d) *Ccg* venom injected mice after 6 hours (x400).

Light microscopy of the kidney sections stained with MBB Stain showed the total proteins in epithelial cells lining the renal tubules of control mice as deeply blue stained diffuse granules homogenously through the cytoplasm and nuclei (Figure 6 a). Their nuclear envelopes and nucleoli as well as some chromatin elements were also positively stained. Injected mice with LD₅₀ of *Ccg* venom for 1 and 3 hours induced a slight decrease in the protein content of the urinary tubules (Figures 6 b and c). More reduction in proteins was manifested in the cells after 6 hours of envenomation, where the proteinic granules were clearly reduced in amount and stainability (Figure 6 d).

**Figure 6:** Sections of kidney stained with bromophenol blue. a) Control mice, b) *Ccg* venom injected mice after 1 hour, c) *Ccg* venom injected mice after 3 hours and d) *Ccg* venom injected mice after 6 hours (x400).

The histochemical examination of testis sections of control group mice stained with MBB Stain showed the presence of moderate content of protein in the form of MBB Stain positive materials all testis tissue (Figure 7a). Injection of mice with LD₅₀ of *Ccg* venom for 1 and 3 hours induced a slight decrease in the protein content of the testis tissue (Figures 7 b and c). More reduction in proteins was manifested in the cells after 6 hours of envenomation (Figure 7d).

**Figure 7:** Sections of testis stained with bromophenol blue. a) Control mice, b) *Ccg* venom injected mice after 1 hour, c) *Ccg* venom injected mice after 3 hours and d) *Ccg* venom injected mice after 6 hours (x400).
3.3. Biochemical results:
In the present work, mice envenomated with Ccg crude venom at LD$_{50}$ dose for the 1, 3 and 6 hours, represented different changes of the selected biochemical parameters. Data showed in Table (1) indicates that Ccg crude venom induced a highly significant elevation in renal MDA level at the 3$^{rd}$ hour at P≤0.001 and a significant increase in hepatic, renal and testicular MDA levels at P≤0.05 at the 6$^{th}$ hour as compared to the control group.

The level of nitrite/nitrate (NO) of envenomated mice was highly significant decreased in hepatic homogenate at the 1$^{st}$ hour (-32.85%) as compared to the control group. On contrast, NO was significantly increased in hepatic homogenate at the 3$^{rd}$ and 6$^{th}$ hours (45.01% and 163.89%, respectively) when compared to the control group.

Table 1: The effect of LD$_{50}$ of crude Ccg i.p injected for 1, 3 and 6 hours on MDA and nitrite/nitrate levels in hepatic, renal and testicular homogenates of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>1$^{st}$ hour</th>
<th>3$^{rd}$ hour</th>
<th>6$^{th}$ hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic MDA (nmol/g tissue)</td>
<td>3.85±0.136</td>
<td>4.65±0.352</td>
<td>5.19±0.136$^*$</td>
<td>5.17±0.339$^*$</td>
</tr>
<tr>
<td>Renal MDA (nmol/g tissue)</td>
<td>4.00±0.072</td>
<td>4.31±0.115</td>
<td>5.27±0.207$^{**}$</td>
<td>4.92±0.130$^*$</td>
</tr>
<tr>
<td>Testicular MDA (nmol/g tissue)</td>
<td>5.36±0.432</td>
<td>5.77±0.136</td>
<td>5.92±0.292</td>
<td>6.79±0.546$^*$</td>
</tr>
<tr>
<td>Hepatic nitrite/nitrate (μmol/ g tissue)</td>
<td>146.62±8.467</td>
<td>98.46±3.959$^*$</td>
<td>212.62±8.229$^*$</td>
<td>386.92±23.945$^{**}$</td>
</tr>
<tr>
<td>Renal nitrite/nitrate (μmol/ g tissue)</td>
<td>102.15±5.975</td>
<td>131.53±8.430</td>
<td>139.23±13.456</td>
<td>240.00±11.701$^{**}$</td>
</tr>
<tr>
<td>Testicular nitrite/nitrate (μmol/ g tissue)</td>
<td>77.66±5.473</td>
<td>69.39±4.181</td>
<td>98.32±5.584$^*$</td>
<td>107.38±4.528$^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE. $^*$: Significant against vehicle control group at P≤0.05, $^*$: Significant at P≤0.01 and $^{**}$: Significant at P≤0.001, n=6.

Table (2) showed that the i.p injection of the Ccg crude venom induced a highly significant reduction in the levels of hepatic GSH at the 1$^{st}$ (-17.24%), 3$^{rd}$ (-21.84%) and 6$^{th}$ hours (-33.33%) after Ccg envenomation as compared to control group. The levels of renal and testicular GSH levels were non-significantly decreased after one hour of Ccg envenomation while the renal and testicular GSH levels after six hours were significantly decreased as a result of LD$_{50}$ envenomation.

Table 2: The effect of LD$_{50}$ of crude Ccg i.p injected for 1, 3 and 6 hours on GSH levels in hepatic, renal and testicular homogenates of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>1$^{st}$ hour</th>
<th>3$^{rd}$ hour</th>
<th>6$^{th}$ hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic GSH (mg/g tissue)</td>
<td>0.87±0.034</td>
<td>0.72±0.026$^*$</td>
<td>0.68±0.047$^{**}$</td>
<td>0.58±0.038$^{**}$</td>
</tr>
<tr>
<td>Renal GSH (mg/g tissue)</td>
<td>0.65±0.027</td>
<td>0.58±0.031</td>
<td>0.47±0.023$^*$</td>
<td>0.46±0.019$^{**}$</td>
</tr>
<tr>
<td>Testicular GSH (mg/g tissue)</td>
<td>0.19±0.049</td>
<td>0.16±0.008</td>
<td>0.15±0.011$^*$</td>
<td>0.12±0.009$^{**}$</td>
</tr>
</tbody>
</table>

Values are means ± SE. $^*$: Significant against vehicle control group at P≤0.05, $^*$: Significant at P≤0.01 and $^{**}$: Significant at P≤0.001, n=6.

The crude Ccg venom injection resulted in a highly significant decrease in CAT activity of hepatic, renal and testicular tissues at P≤0.001 after 1, 3 and 6 hours as shown in Figure (8).
congestion, and multifocal areas of ischemic necrosis of spermatogenic cells, oedema, haemorrhage, testicular tissue in the form of degeneration of have shown marke

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1966) and cytotoxicity (Bertke and Atkins, 1961). Common

and Khaled, 1966), nephrotoxicity (Ickowicz et al., 1997; Mahmoud, 1983), muscular dystrophy (Mohamed Sinna et al., 1993), general metabolic disturbance of

initial signs of envenoming are hypoglycemia (Abu enzymes (Tan and Ponnudurai, 1990). Common

viper venoms are due to their proteolytic and lipolytic

effect of snake venom for pathophysiological conditions is known for centuries (Pal et al., 2002). Several works dealing with the effects of snake venoms 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 (Maria et al., 2003; Fox and Serrano, 2008). So, this work was undertaken to study the effect of Cerastes cerastes gasperetti (Ccg) crude venom on histological, histochemical changes and on oxidative stress status in hepatic, renal and testicular homogenates.

Snake venom components, especially those of viper venoms, activate, inhibit or liberate enzymes by destroying cellular organelles (Abdel-Nabi et al., 1997; Marsh, 1997). The different toxic effects of viper venoms are due to their proteolytic and lipolytic enzymes (Tan and Ponnudurai, 1990). Common initial signs of envenoming are hypoglycemia (Abu-Sinna et al., 1993), general metabolic disturbance (Mahmoud, 1983), muscular dystrophy (Mohamed and Khaled, 1966), nephrotoxicity (Iekowicz et al., 1966) and cytotoxicity (Bertke and Atkins, 1961). The local effects of the snake bite include: oedema, haemorrhage, dermonecrosis and myonecrosis (Chippaux et al., 1991; Tu, 1996; Kini, 1997; Shashidhara-Murthy et al., 2002; Cher et al., 2005) and in agreement with these results, the current study has shown marked histological changes in the testicular tissue in the form of degeneration of spermatogenic cells, oedema, haemorrhage, congestion, and multifocal areas of ischemic necrosis after Ccg crude venom injection at the all selected time intervals.

Renal failure can be expected after envenoming by vipers including Cerastes cerastes with a serious course of intoxication. The underlying mechanisms are, besides glomerular hypofiltration by bleeding, decrease of intravascular volume by extravasation, microthrombi formation within consumption coagulopathy and vasoconstriction and direct nephrotoxicity of venom constituents: enzymatic destruction of renal marrow and renal tubules (Warrell, 1995).

Regarding the histochemical changes observed in this study after Ccg crude venom injection, results clearly indicated reduction in the polysaccharides and total proteins in the liver, kidney and testis tissues. These changes were consistent with those induced histopathologically in liver and kidney tissues by the same venom (Al–Sadoon et al., 2013).

The decrease in carbohydrate content was attributed by some investigators to be due to increased stress on organs, leading to high energy consumption which allowed an equalized pressure to be exerted upon them. The decrease in protein could be attributed to the disruption of lysosomal membranes under the effect of various toxicants leading to the liberation of their hydrolytic enzymes in the cytoplasm resulting in marked lysis and dissolution of the target material. This result confirmed that of Awasthi et al. (1984), who found elevated lysosomal enzymatic activity accompanied by a decrease in protein and nucleic acids content in response to organophosphate insecticide.

In the present study, the envenomation of mice with Ccg crude venom after 1, 3 and 6 hours showed a highly significant increase in lipid peroxidation and nitrite/nitrate levels as compared with control group. However, Ccg crude venom injection induced a highly significant reduction in levels of GSH and CAT activity after the three selected time intervals. Oxidative stress may be a result of excessive reactive oxygen species generation or failure of the cellular antioxidant system. Snakes venom induced an elevation of oxidative stress indicators as nitric oxide and lipid peroxidation (Al Asmari et al., 2006). Glutathione is widely distributed tripeptide and found mainly in the cell cytosol (Mitchell and Jollows, 1975). Glutathione is the cell’s natural antioxidant, which destroys free radicals formed in cells. This plays a crucial role in the detoxification process. Our results supported by the previous interpretation of the consequences of the GSH deficiency which causes oxidant damage and greater lipid peroxidation which in turn leading to cell damage (Wang et al., 2000; Scholz et al., 1997; Bouchard et al., 2000).
It is clear from this study that, Ceg crude venom has drastic toxic effects on liver, kidney and testis tissues as represented by the observed histochemical and biochemical changes. So our future goal is to characterize the relative role of some medicinal plants and natural products on neutralizing or modulating these toxic effects of the Ceg crude venom.

5. Conflict of interest
The authors declare that there are no conflicts of interest.

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