The possible effects of melatonin in *Cerastes cerastes gasperettii* venom-mediated toxicity and oxidative damage in mice

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In this study, the role of melatonin has been explored for the prevention of *Cerastes cerastes gasperettii* venom-mediated toxicity and oxidative injury in male mice. The horned viper, *C. c. gasperettii*, is the most common snake in the subtropical deserts, including Saudi Arabia; its bite can be fatal. Therefore, we investigated the potential role of melatonin (10 mg/kg body wt) against the LD50 of *C. c. gasperettii* venom (0.978 mg/kg body wt) after 1, 2 and 6 h. The *in vivo* oxidative injury induced by the venom was clearly evident by the increased oxidative stress markers (lipid peroxidation and nitrite/nitrate) and decreased antioxidant molecules/enzymes (glutathione, superoxide dismutase and catalase), along with the histopathological alternations in liver, kidney and lung. In addition, mice envenomation disturbed the normal serum levels of liver and kidney function parameters. These biochemical changes and pathological alternations prior to *C. c. gasperettii* venom injection were effectively counteracted by melatonin. In conclusion, these results clearly indicate that melatonin attenuates oxidative stress, pathogenesis and dysfunction of liver, kidney and lung of mice in *C. c. gasperettii* venom-induced toxicity and oxidative injury via its antioxidant property. Therefore, it may be useful for the treatment for *C. c. gasperettii* bites.

Keywords: *Cerastes cerastes gasperettii*, melatonin, oxidative damage, venom-mediated toxicity.

SNAKEBITE is one of the most important public health problems of tropical and subtropical countries[1,2]. It has been reported that globally over one million people are bitten annually by numerous snakes, resulting in 70,000 deaths. The major pathophysiological problems associated with snakebites generally start with systemic side effects like nausea, vomiting, headache, diarrhoea, abdominal pain, fall in blood pressure, etc. followed by late systemic effects like neuromuscular blockage and haemorrhage. Snake envenomation is associated with local side effects like pain, swelling and necrosis, and usually these effects appear rapidly prior to the snake bite if the serotherapy is delayed due to either late access to medical care or scarcity of the specific antivenoms[3].

Furthermore, a complex array of venom cytotoxins penetrates the cells and interacts with intracellular targets, such as the mitochondria[4]. Mitochondrial impairment can generate reactive oxygen/nitrogen species (ROS/RNS) that in turn reduce mitochondrial bioenergetics, leading to cell damage and accerelate cell death[5]. The persistence of oxidative stress injures vital organs such as liver and kidneys, resulting in multiple organ failure.

In the present study, *Cerastes cerastes gasperettii* was selected based on its medical importance in Saudi Arabia. The major toxins found in the venom of *C. c. gasperettii* are snake venom metalloproteinases (SVMPs), phospholipase A_2s (PLA_2s), C-type lectins (CTLs), disintegrins (DISs), cysteine rich secretory proteins (CRISPs), and L-amino acid oxidases (LAAOs)[6]. SVMPs are responsible for local haemorrhage, extracellular matrix degradation, blistering and skin necrosis. In addition, both PLA_2s and SVMPs promote an inflammatory response that sets the stage for tissue repair and regeneration but, at the same time, may contribute to further tissue damage[7]. Moreover, LAAOs are thought to contribute to venom toxicity, possibly through forming H_2O_2 (ref. 8).

Antiserum contains antibodies against some of the snake venom components, and is the only agent available to treat envenomation. It appears to ameliorate some of the toxic effects of venom, but fails to provide protection against venom-mediated necrosis, haemorrhage and renal failure[5]. Moreover, antiserum is often associated with hypersensitivity reactions that may be lethal to the victim[9]. Furthermore, antiserum is expensive and thus out of reach of many patients in rural areas. Finally, lack of information about the biting species in most cases, and the correct antiserum dosage and stability restrict its efficacy[5]. Therefore, it is of clinical and economic importance to find new and effective therapies for snakebite.

Melatonin (Mel) is a potent free-radical scavenger with an antioxidant property. Mel can directly detoxify

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ROS/RNS[10,11]. It also acts as an indirect antioxidant by regulating the activities of enzymes which promote overall the antioxidative defence systems in an organism, including glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase[12]. In addition, the metabolites, including N1-acetyl-N2-formyl-5-methoxykynuramine and N1-acetyl-5-methoxykynuramine, formed by the interaction of Mel with free radicals are efficient radical scavengers[13]. Therefore, the present study was aimed to investigate the potential role of melatonin against the lethal dose 50% (LD_{50}) of *C. c. gasperettii* venom-mediated toxicity and oxidative injury in the liver, kidney and lung of mice after 1, 2 and 6 h of envenomation.

Materials and methods

Snake venom

The horned viper venom collection and preparations: The crude venom was obtained by milking about 20 samples of *C. c. gasperettii* collected from Al-Qassim, the central region of Saudi Arabia (26°–27°N lat., 44°–45°E long.). The approximate LD_{50} of the crude venom was calculated in our previous study[14] to be 0.978 mg/kg body wt, of mice according to the method described by Meier and Theakston[15].

Experimental animals

Experiments were performed on adult 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. They 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. The mice were supplied with tap water and balanced diet *ad libitum*. They were acclimatized for one week prior to experimental use. The experiments were approved by the local state authorities and were performed in compliance with the Egyptian rules on animal protection.

Experimental protocol

To study the potential role of melatonin on *C. c. gasperettii* crude venom-mediated toxicity and oxidative injury, 63 mice were randomly divided into groups as follows:

Control group (group I, $n = 21$): mice were injected with 0.2 ml saline solution/mouse intraperitoneally (i.p.) and were sacrificed after the first, second and sixth hour of injection.

LD_{50} dose envenomated group (group II, $n = 21$): mice were i.p. injected with 0.2 ml saline solution containing 0.978 mg/kg body wt and were sacrificed after the first, second and sixth hour of injection.

Group III ($n = 21$): mice were injected i.p. with melatonin at a dose of 10 mg/kg body wt 10 min after the *C. c. gasperettii* venom injection at LD_{50} dose, and were sacrificed after the first, second and sixth hour of injection. The dosage of the administered melatonin (10 mg/kg body wt) was chosen according to the work of El-Sokkary *et al.*[16].

All the animals were sacrificed by fast decapitation. Blood samples were collected, allowed to stand for 30 min and then centrifuged at 1000 *g* (15 min, 4°C). Blood serum was collected and stored at –20°C for different biochemical measurements. Liver, kidney and lung were collected and pieces of each organ were fixed in 10% neutral buffered formalin for histopathological investigations. Some parts from the liver, kidney and lung were also weighed and homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 1000 *g* (10 min, 4°C) and the resulting supernatant was stored at –20°C until use in the various biochemical determinations.

Biochemical studies

Liver and kidney function tests: Colorimetric estimation of transaminases (alanine aminotransferease (ALT) and aspartate aminotransferase (AST)) was made by determining the quantity of pyruvate or oxaloacetate produced by forming 2,4-dinitrophenylhydrazine, according to the method of Reitman and Frankel[17]. γ-Glutamyltransferase (γGT; CAT. NO. GT 523) and alkaline phosphatase (ALP; CAT. NO. AP 311) were assayed using kits provided by Randox Laboratories Co, UK. Total bilirubin (TB), uric acid, urea and creatinine levels were assayed by commercially available diagnostic kits (Diamond diagnostics, USA) according to the manufacturer's instructions.

Oxidative stress markers: Nitrite/nitrate (nitric oxide; NO) and lipid peroxidation (LPO) levels were determined colorimetrically in the liver, kidney and lung homogenates according to the methods of Green *et al.*[18] and Ohkawa *et al.*[19] respectively. Similarly, the liver, kidney and lung glutathione (GSH) contents were determined by the method of Ellman[20].

Superoxide dismutase and catalse activity: The activity of SOD in the liver, kidney and lung was determined by the method of Nishikimi *et al.*[21]. In addition, catalase (CAT) activity was measured in homogenates of liver, kidney and lung following the methodology of Aebi[22].

Table 1. Effect of LD_{50} of crude venom of *C. c. gasperettii* and melatonin injections after 1, 2 and 6 h on liver and kidney functions in serum of mice

		First hour		Second hour		Sixth hour	
Group	Group I	Group II	Group III	Group II	Group III	Group II	Group III
ALT (U/ml)	44.40 ± 0.82	52.78 ± 2.18[a]	50.02 ± 3.76[a]	82.74 ± 4.22[a]	61.34 ± 2.69[ab]	98.75 ± 3.18[a]	69.64 ± 4.21[ab]
AST (U/ml)	52.82 ± 0.65	71.43 ± 5.28[a]	66.42 ± 3.22[ab]	88.03 ± 4.97[a]	71.54 ± 4.75[ab]	114.36 ± 8.20[a]	77.83 ± 5.32[ab]
ALP (IU/l)	59.30 ± 1.46	70.05 ± 3.66[a]	62.38 ± 2.31[b]	50.08 ± 1.42[a]	58.74 ± 3.81[b]	35.18 ± 2.33[a]	50.54 ± 5.71[ab]
γGT (U/l)	2.86 ± 0.16	4.74 ± 0.13[a]	3.05 ± 0.43[b]	5.08 ± 0.08[a]	3.46 ± 0.34[ab]	5.82 ± 0.11[a]	4.01 ± 0.32[ab]
Total bilirubin (mg/dl)	3.45 ± 0.17	4.13 ± 0.13[a]	3.87 ± 0.22	4.91 ± 0.21[a]	4.02 ± 0.27[ab]	5.68 ± 0.31[a]	4.48 ± 0.40[ab]
Creatinine (mg/dl)	0.23 ± 0.01	1.21 ± 0.11[a]	0.82 ± 0.10[ab]	3.32 ± 0.25[a]	2.25 ± 0.22[ab]	8.87 ± 0.52[a]	5.84 ± 0.86[ab]
Urea (mg/dl)	25.20 ± 1.82	57.53 ± 4.37[a]	42.21 ± 4.12[ab]	85.43 ± 5.63[a]	63.43 ± 5.81[ab]	134.51 ± 9.64[a]	101.38 ± 11.34[ab]
Uric acid (mg/dl)	12.07 ± 0.11	8.11 ± 0.72[a]	8.37 ± 0.64[a]	4.23 ± 0.32[a]	5.56 ± 0.63[ab]	2.36 ± 0.16[a]	3.03 ± 0.21[ab]

Values are mean ± SEM (n = 7). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

Histopathological studies

Tissue samples of liver, kidney and lung were fixed in 10% neutral formalin for 24 h, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58–62°C. Slices of 4–5 µm were obtained from the prepared blocks and stained with haematoxylin–eosin. The preparations obtained were visualized using a Nikon (Eclipse E200-LED, Tokyo, Japan) microscope at 400× magnification.

Statistical analysis

Results were expressed as mean ± SEM. Data for multiple variable comparisons were analysed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's post hoc test was used (SPSS version 17.0).

Results

Cellular integrity

Table 1 gives the levels of serum transaminase (ALT, AST), in addition to those of ALP, γGT and TB in the control and envenomated mice. Envenomated mice had significant ($P < 0.05$) increased levels of ALT, AST, γGT and TB in the serum after 6 h of *C. c. gasperettii* venom injection. Melatonin, on the other hand, was able to reduce these elevations. However, melatonin could not return it to the control values. The increments in those parameters were associated with significant ($P < 0.05$) inhibition in ALP activity after 6 h of *C. c. gasperettii* venom injection in mice. Furthermore, intoxicated group showed significant ($P < 0.05$) increased levels of creatinine, urea and uric acid (Table 1). Treatment of mice with melatonin had reversed these parameters. However, melatonin again failed to return these to the control levels. Melatonin injection per se did not cause significant changes in liver and kidney function parameters (data not shown).

Oxidative stress results

It is evident from Figures 1 and 2 that envenomated mice have significantly ($P < 0.05$) higher values of NO and LPO in the liver, kidney and lung homogenates. Venom-induced changes in NO and LPO levels are ameliorated significantly in the melatonin post-treated mice compared to the envenomated mice. It is clear from the results that the NO and LPO contents in injected melatonin groups are near the normal values. Melatonin injection alone in mice causes significant decrease in LPO level in all examined tissues, and NO level in liver and kidney (data not shown).

Oxidative stress in tissues generally involves the GSH system; therefore, the GSH content in each group was measured. Envenomated mice have significantly ($P < 0.05$) lower GSH content in liver, kidney and lung tissues (Figure 3). It was observed that melatonin treatment after envenomation increased the GSH content significantly ($P < 0.05$) as compared to intoxicated group, thereby indicating its protective role against toxicity.

Envenomation with *C. c. gasperettii* in mice caused overproduction of cellular oxidants and modulation of antioxidant defence system. As observed during the study, venom injection led to modulation of several parameters of oxidative stress relative to the control animals. SOD activity in the different examined homogenates of liver, kidney and lung tissues decreased significantly ($P < 0.05$) as compared to the control (Figure 4). On the other hand, melatonin post-treatment significantly elevated the SOD ($P < 0.05$) compared to *C. c. gasperettii* group. Moreover, melatonin post-treatment significantly elevated SOD content in different homogenates of liver, kidney and lung tissues as compared to the control.

C. c. gasperettii snake venom injection in mice caused significant ($P < 0.05$) reduction in CAT activity in all tested organs. Again, mice injected with 10 mg/kg body wt of melatonin post-treatment to snake venom injection had significantly ($P < 0.05$) elevated CAT activity compared to *C. c. gasperettii* injected group (Figure 5). As expected, melatonin injection alone in mice caused

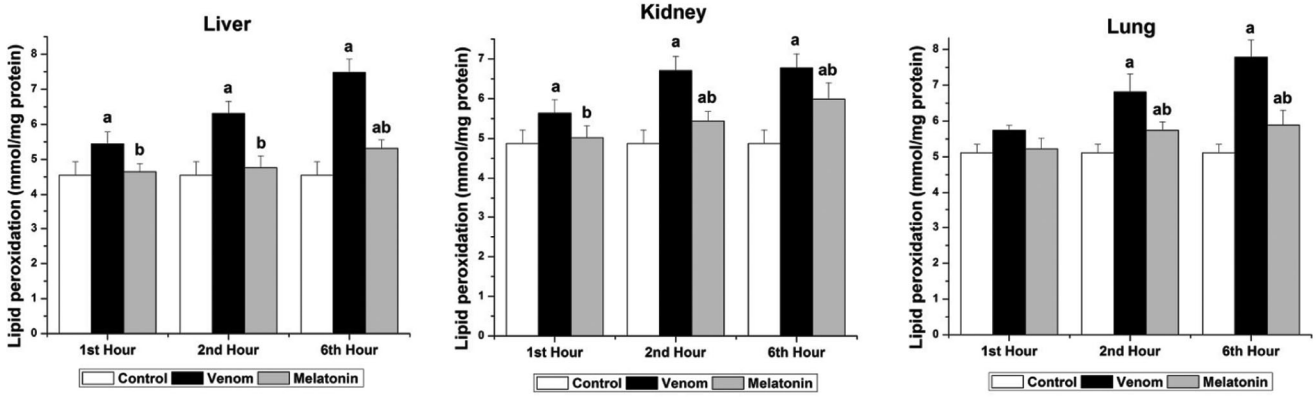

Figure 1. Lipid peroxidation level in liver, kidney and lung tissues of male mice injected with *Cerastes cerastes gasperettii* venom and melatonin. Values are mean ± SEM ($n = 7$). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

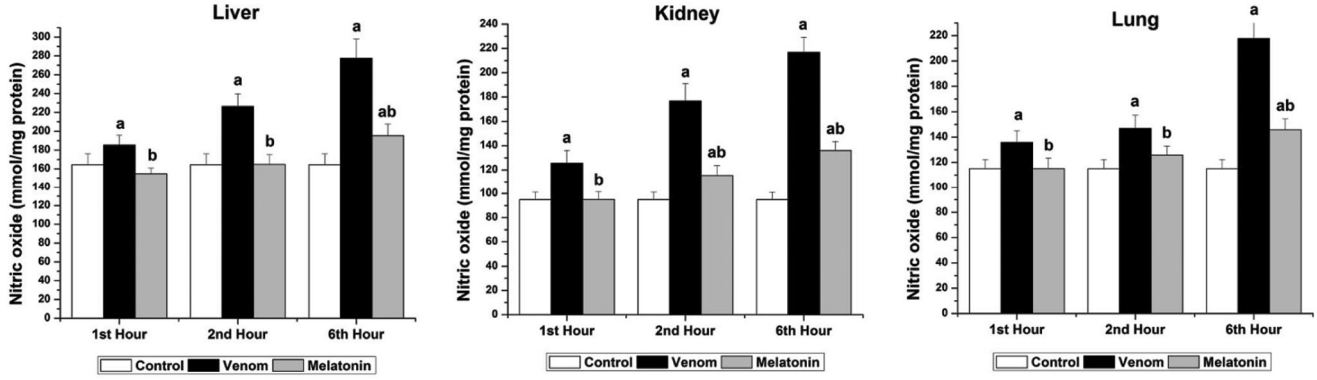

Figure 2. Nitric oxide level in liver, kidney and lung tissues of male mice injected with *C. c. gasperettii* venom and melatonin. Values are mean ± SEM ($n = 7$). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

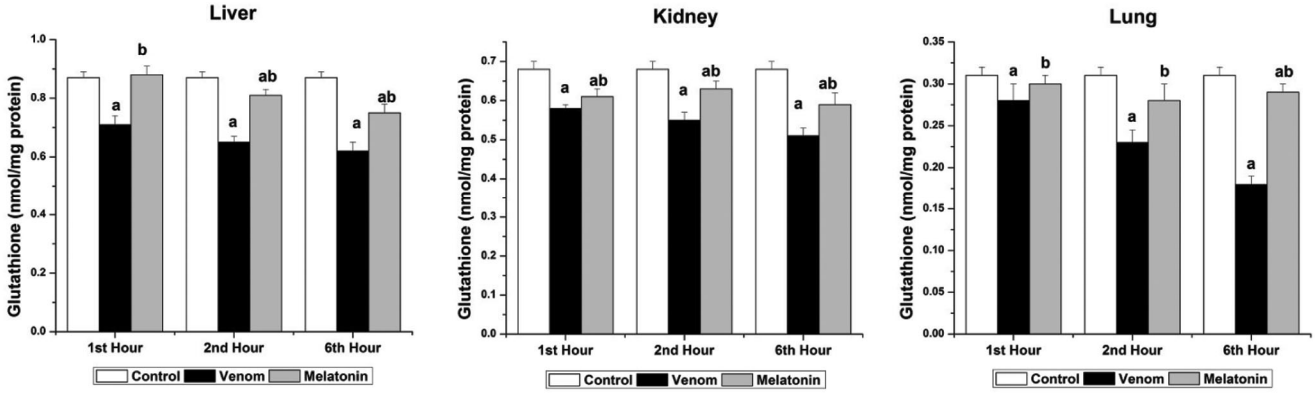

Figure 3. Glutathione content in liver, kidney and lung tissues of male mice injected with *C. c. gasperettii* venom and melatonin. Values are mean ± SEM ($n = 7$). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

significant increase in antioxidant molecules/enzymes (data not shown).

Histopathogy results

Liver sections of mice injected with LD_{50} of *C. c. gasperettii* crude venom appeared with inflammatory cellular infiltrations around the hepatic vein. Also, hepatic sinusoids were dilated and contained prominent van Kupffer cells (Figure 6). Severe necrosis and apoptosis were also seen after the injection of *C. c. gasperettii* venom; the degree of damage is time-dependent. Melatonin could repair these induced effects (Figure 6).

Microscopic examination of the renal tissue showed that the venom induced severe glomerular degeneration and coagulative necrosis. Also, the urinary spaces

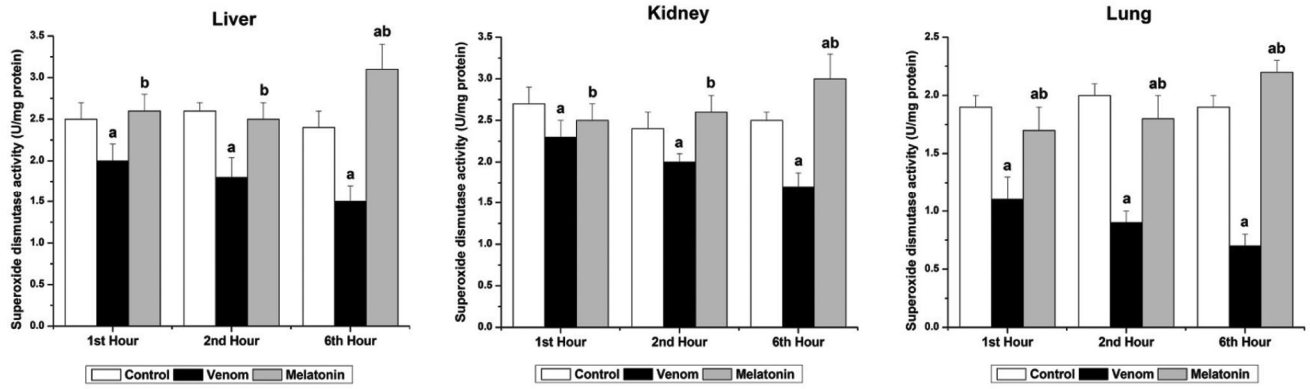

Figure 4. Superoxide dismutase activity in liver, kidney and lung homogenates of male mice injected with *C. c. gasperettii* venom and melatonin. Values are mean ± SEM ($n = 7$). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

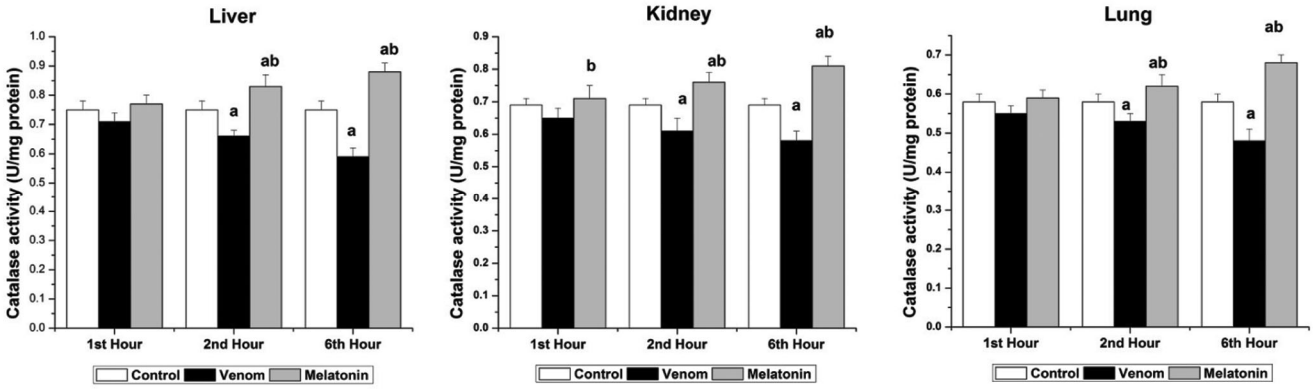

Figure 5. Catalase activity in liver, kidney and lung homogenates of male mice injected with *C. c. gasperettii* venom and melatonin. Values are mean ± SEM ($n = 7$). [a]$P < 0.05$, Significant change with respect to control group. [b]$P < 0.05$, Significant change with respect to venom group for Duncan's post hoc test.

appeared wider compared to the control. Moreover, most of the renal tubules had degenerated and were filled with cellular debris (Figure 6). Post-treatment of melatonin prevented renal histo-architecture from most alteration and displayed well-formed glomerulus in Bowman's capsule (Figure 6).

Also, extensive diffuse haemorrhagic areas were found in all lung lobes of animals in the experimental injected groups. Histological analysis of the lung tissue of envenomated animals showed moderate congestion of the alveolar capillaries and large quantities of erythrocytes distributed diffusely in the interstitial and intra-alveolar spaces (Figure 6); the degree of these histo-damages is time-dependent. Such haemorrhagic indications demonstrate the direct action of the venom. Post-treatment with melatonin reversed these changes to near normalcy.

Discussion

Snake envenomation is a serious public health problem not only in the Arabian countries, but also in different tropical and subtropical regions of the world. This is due to its potential to induce severe and sometimes terminal damages in several organs of the body. The venom induces several clinical signs and symptoms ranging from local, autopharmacological, antihemostatic, neurological, muscular, cardiac to renal effects[23,24]. Although much work has been done to determine the exact pathophysiology that leads to these venom-evoked effects, more studies are needed to establish the exact mechanisms involved, especially at the cellular level. Since death due to snake envenoming is usually due to respiratory, hepatic and renal complications[25], and since the liver, kidney and lung are readily susceptible to the deleterious effects of oxidative stress-generated free radicals[26], oxidative stress markers have been measured in these vital organs of envenomated mice.

Liver is a major producer of most of the serum proteins. In the present study, the elevation in serum ALT, AST, γGT, ALP and TB levels in *C. c. gasperettii* envenomated mice could be attributed to damage to the hepatocytes, together with haemorrhages in vital organs as heart and muscles. Consequently, there will be discharge of the cell contents into the blood stream, as reported by

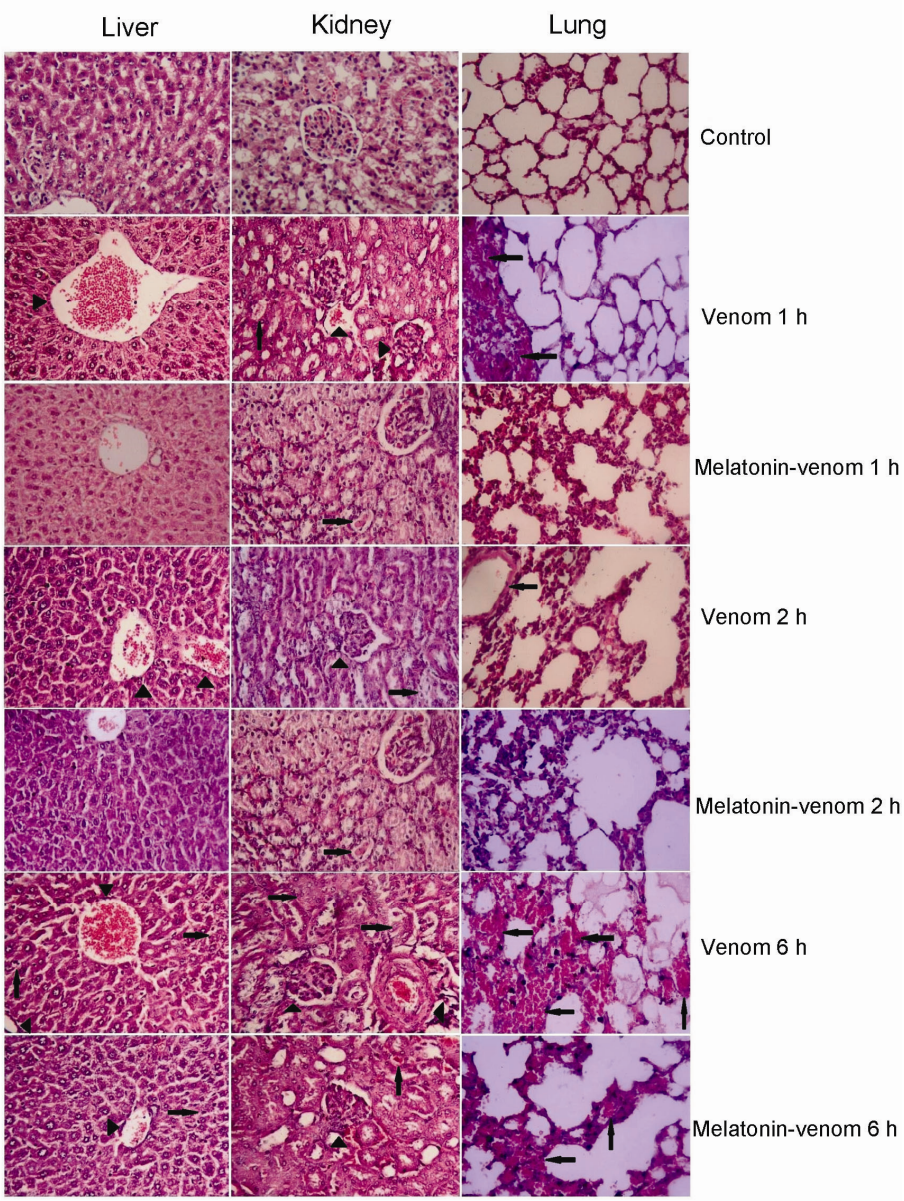

Figure 6. Photomicrographs of haematoxylin–eosin staining of hepatic, renal and lung tissues of control *C. c. gasperettii*, and *C. c. gasperettii* venom and melatonin-treated mice (40× magnification). Inflammatory cellular infiltrations around the hepatic vein (►) and severe necrosis and apoptosis (arrow) are seen in *C. c. gasperettii* venom hepatic section. In renal sections, glomerular degeneration, coagulative necrosis (►) and renal tubules are seen to be degenerated and filled with cellular debris (arrow). In lung sections, congestion of the alveolar capillaries and large quantities of erythrocytes distributed diffusely in the interstitial and intra-alveolar spaces (arrow) are the most predominant alternations.

other studies on snake venom[7,8]. In addition, the increase in vascular permeability and haemorrhages in the vital organs due to the toxic action of various snake venoms has been described[27,28]. In the present study, we explored the systemic physio-pathological changes induced by *C. c. gasperettii* venom on kidney as a vital organ. The elevated levels of creatinine, urea and uric acid in the serum indicate renal dysfunction. Similar observations were reported in rats following administration of various snake venoms[29,30]. Melatonin significantly reduces liver and kidney injuries and acts as a hepatorenal protective agent.

In order to study the involvement of free-radical generation and oxidative stress in venom-induced cellular damages, the free-radical scavenger, melatonin, was intrapritoneally injected to mice 10 min after *C. c. gasperettii* venom injection. Melatonin was chosen since it is a readily available, safe natural product that has proven to be effective in combating oxidative stress resulting from different conditions such as ischaemic/reperfusion injuries[31], oxidative stress-mediated platelet aggregation[32], and acute hypoxic stress[33].

In the present study, *C. c. gasperettii* venom increased both LPO and NO levels in the liver, kidney and lung of

mice. This demonstrates the role played by *C. c. gasperettii* venom to induce tissue toxicity. The venom-evoked enhanced LPO and free-radical generation observed in this study may elucidate the deleterious organ defects and multiple organ disorders observed following snake envenomation[14].

Snakebites are most often accompanied by signs of inflammation and local tissue damage. Neutrophils and macrophages are stimulated to produce superoxide radical anion which belongs to a group of ROS. This reacts with cellular lipids leading to the formation of different peroxides and subsequently leading to tissue necrosis. As the origin of oxidative stress is the mitochondrial respiratory electron transport chain, it is possible that mitochondrial death potentiates venom-induced cellular damage[34].

High levels of LPO and NO are indicators of oxidative stress. The oxidative stress and alteration of protein contents suggest membrane destruction, enzyme release and protein loss due to envenomation[24]. Oxidative stress may occur when the balance that exists between the formation of ROS and their removal by endogenous antioxidant scavenging compounds is disrupted by excessive production of ROS. Alternatively, oxidative stress may also be caused by inappropriate antioxidant defences due to changes in SOD and CAT activities and glutathione levels[35].

A very large body of evidence indicates that melatonin is a major scavenger of both oxygen- and nitrogen-based reactive molecules, including $ONOO^-$ (ref. 36). Melatonin has scavenging actions at both physiological and pharmacological concentrations. Not only melatonin, but also several of its metabolites can detoxify free radicals and their derivatives[37]. Studies have also revealed that melatonin eliminates the decomposition products of $ONOO^-$ and $CO_3^{\bullet -}$ (ref. 38). Melatonin also supports several intracellular enzymatic antioxidant enzymes, including SOD[39,40].

SOD is one of the main antioxidant defence enzymes activated in response to oxidative stress. SOD detoxifies the highly toxic superoxide anions by converting them into hydrogen peroxide (H_2O_2). In the present study, SOD activity was significantly inhibited in the liver, kidney and lung of *C. c. gasperettii* venom-injected mice, as also reported by Venkatesan *et al.*[41]. However, significant increase in SOD activity was observed in *C. c. gasperettii* venom-injected mice post-treated with melatonin. Several studies have revealed that melatonin increases the mRNA expression of copper–zinc SOD and manganese SOD[39,40]. These stimulatory effects were observed after both acute and chronic melatonin administration. Melatonin may act directly at a genetic level or via an indirect pathway to activate SOD.

The inhibition of CAT after venom inoculation is probably due to oxidative stress related to the release of free iron that catalyses ROS production, initiating LPO formation and increasing the redox imbalance in different organs. We present evidence that CAT is also the target of the venom, and it can be an important agent driving oxidative stress during *C. c. gasperettii* intoxication. Our data show that the injection of melatonin in mice encourages CAT activity. Mechanisms responsible for the beneficial effects of melatonin and/or its metabolites include a reduced electron leakage and ROS generation, protection of macromolecules from oxidative stress and increased activity of antioxidant enzymes[42].

In general, post-injection of snake envenomated mice with melatonin significantly ameliorates the venom-evoked increases in LPO and NO levels in rat liver, kidney and/or lung, indicating the involvement of free-radical generation, oxidative stress and organ damage in the deleterious effects of the venom in the liver, kidney and lung. Melatonin may also have beneficial effects in snake envenomation, such as its vasodilating capability[43], which would be useful in cases of venom-induced myocardial ischaemia and infarction[31].

Our data have shown that *C. c. gasperettii* venom exhibits deleterious effects on the lung, liver and kidney. These concern mainly the haemolytic and oxidative stress induced after *C. c. gasperettii* injection. The action of the crude venom is probably the result of the concomitant action of several components. From a complex mixture, one can expect synergism and even antagonism. Further studies with purified components of *C. c. gasperettii* venom are needed to obtain detailed information about its mechanisms of action and its potential biotechnology applications. The present data are, therefore, more relevant for the clinical aspects of *C. c. gasperettii* intoxication.

In conclusion, the efficiency of the antioxidant, melatonin, in attenuating venom-evoked changes indicates the involvement of oxidative stress in venom-mediated cellular damages. Additionally, the use of antioxidants that could exert their protective actions within a short period of time would be more convenient and should be investigated. Thus, melatonin, if proven to be effective in combating snake venom-mediated cellular damages, may have a future role in the treatment protocol of snake envenomation.

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