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Pro-inflammatory cytokines gene expression in liver and kidneys of rats exposed to a sub-lethal dose of *Bitis arietans* snake venom

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Abstract

Bitis arietans (Puff adder) is a poisonous snake and its bite causes pain, edema, blistering, tissue damage and neutrophilia. There are limited studies on inflammatory process involved in *Bitis arietans* envenomation. We therefore investigated the role of proinflammatory cytokines in *Bitis arietans* venom (BAV)-induced liver and kidney toxicities in rats. Adult male Sprague Dawley rats were treated with BAV (0.5 mg/kg) and were sacrificed after specific time intervals (2 h, 24 h, 1 week). Blood samples were collected for liver and renal function tests and tissues were collected for histopathology and gene expression analysis of IL-1β, IL-6, and TNF-α in liver and kidneys. There was no significant difference in serum ALT activities among different treatment groups. Serum AST was significantly increased at 24 h following BAV injection. In both organs, injection of BAV resulted in mild inflammatory cell infiltration at 2 h post-dosing which normalized after 1 week. In liver, there was a significant increase in IL-1β expression in BAV-treated rats at 2 and 24 h post-dosing that reduced after one week. Significant increases in IL-6 and TNF-α were observed at 24 h and 1 week after BAV exposure. In kidneys, there were significant increases in IL-1β and TNF-α expression at 24 h that subsided after 1 week. In conclusion, a single sub-lethal dose of BAV caused an acute phase inflammation in liver and kidneys. It is most probable that a higher dose of BAV may result in greater and irreversible damage to these organs.

Keywords: Puff adder; *Bitis arietans*; Snake bites; Inflammation, Proinflammatory cytokines; Liver; Kidney; Toxicity

1. Introduction

Morbidity and mortality due to snakebite is an important socio-medical problem throughout the globe, particularly in rural areas. There are at least ten species of terrestrial venomous snakes inhabiting in Saudi Arabia [1, 2]. The puff adder (*Bitis arietans*) is a venomous viper species that occupies densely populated habitats throughout the Middle East and savannah areas of sub-Saharan Africa [3]. *Bitis arietans* is responsible for a larger number of snake bites in humans and domestic animals than all other African snakes put together [4]. The genus Bitis comprises 17 snake species that inhabit Africa and the Arabian Peninsula. They are responsible for a significant proportion of snakebites in the region due to many factors, such as their wider distribution, frequent occurrence in highly populated areas, and aggressive behavior [5, 6]. The local effects of B. arietans envenomation include swelling, blistering, arterial thrombosis, bruising and necrosis whereas the systemic effects of its envenomation in humans mostly include hypertension, bradycardia, spontaneous bleeding and thrombocytopenia [7]. The mortality due to B. arietans envenomation is rare. However, the lack of early intervention with anti-venom may result in poor quality of life due to disabilities caused by local necrosis [8].

Bitis arietans venom (BAV) contains multiple toxins and is considered as the most toxic venom from any viper species with an LD_{50} of 9–13 µg (approx. 300-430 µg/kg bodyweight) in mice. Previous studies on B. arietans venom have resulted in the isolation of toxins including bitanarin, a novel post-synaptic neurotoxin with PLA_2 activity [3], bitiscetin, a platelet aggregation inducer [9], and Ba100, a toxin with fibrogenase activity [10]. These studies indicate that puff adder as an extremely hemotoxic venom that prevents the formation of platelets in the bloodstream, causing hemorrhage and tissue necrosis. Because of the antivenin crisis in many countries, the puff adders have the potential to cause significant harm to humans they attack with their lethal venom [11].

Studies on the mechanism of action of BAV are crucially important for the development of effective antidotes and pharmacotherapies. Notwithstanding the prevalence of snake bites and fatality of B. arietans envenomation, the underlying mechanisms of toxicity remain poorly understood, particularly regarding the involvement of pro-inflammatory cascade, which can significantly potentiate local [12, 13] and systemic reactions [8, 14]. Recently, Megale et al [4] studied the inflammatory response related to the B. arietans envenomation using a peritonitis mice model.

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They performed most of the biochemical and molecular analyses in peritoneal exudate cells instead of vital organs. In this study, we investigated the role of inflammatory mediators in *Bitis arietans* venom (BAV)-induced liver and kidney toxicities in rats using biochemical, molecular and histopathological approaches.

2. Materials and Methods

2.1. Animals and treatment

Healthy adult male Sprague Dawley rats weighing between 170-180 g were used in this study. The animals were divided into four different groups (N=6 in each group) and labeled by numbers on tails, housed in clean cages and placed in the animal care room with controlled temperature and 12 h light/dark cycles. The rats were allowed free access to food (Purina rodent chow) and tap water *ad libitum*. Control rats received sterile normal saline whereas the remaining three groups were treated intraperitoneally (IP) with *Bitis arietans* venom (BAV) at a dose of 0.5 mg/kg, dissolved in normal saline. The animals in BAV groups were sacrificed at different time intervals; 2 h, 24 h, and 1 week post-dosing, respectively.

Blood samples were obtained by cardiac puncture and collected in serum separator tubes. Tubes were kept at room temperature for 30 minutes and then centrifuged at 1500 g for 10 minutes. The upper layer (serum) was transferred to a new clean tube and stored in a freezer at -80°C (Thermo Scientific, US). Liver and kidneys were dissected out, washed with normal saline, and cut into small pieces before immersing in RNA Later solution (Qiagen, DE) to prevent RNA degradation. For histopathology, liver and kidney samples were fixed in 10% formalin.

2.2. Serum biochemistry

The biomarkers of liver function including serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by commercially available kits (United Diagnostic Industries, Saudi Arabia) following the protocols provided in the kits inserts. Both ALT and AST are liver enzymes and their elevated levels in blood indicate liver injury. For renal function test, the biomarkers including blood urea nitrogen (BUN) and serum creatinine (SCr) were determined by colorimetric kits purchased from United Diagnostic Industries, Saudi Arabia. The retention of BUN, Scr and other metabolic waste products excreted by kidneys indicates acute renal injury.

2.3. Gene expression analysis

Total RNA was extracted from liver and kidney samples (preserved in RNA Later solution) using SV Total RNA Isolation System (Promega, US). In a sterile microcentrifuge tube, approximately 30 mg tissue was taken and 175 µl of RNA Lysis Buffer (containing beta mercaptoethanol) was added and then homogenized by Tissue Tearor Homogenizer (Cole Parmer, USA). This homogenate was used for total RNA extraction using multiple steps of reagent additions and centrifugations, according to prescribed protocol by the Kit supplier. The extracted RNA through spin columns was finally dissolved in 100 µl nuclease-free water and stored at –80°C (Thermo Fisher Scientific, USA). The purity and concentration of RNA was determined by using a Nano-drop Lite Spectrophotometer (Thermo Scientific, USA).

For real time PCR analysis, Power SYBR Green RNA-to-CT One-Step Kit (Applied Biosystems, USA) was used. The reaction mixture (20 µl) contained forward and reverse primers (0.5 mL each), RNA (1 µl), master mix (10 µl), reverse transcriptase enzyme (0.16 µl), and remaining nuclease-free water. The 96-well microplate was incubated at 50°C for half an hour followed by 95°C for 10 min in a real-time PCR instrument. The PCR cycling conditions were as follows: 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 1 min [15]. The primer sequences for the target genes (IL-1β, IL-6 and TNF-α) and the housekeeping gene (GAPDH) are given in Table 1.

2.4. Histopathology

The pre-fixed tissues were processed for the sequential steps of washing, dehydration, clearing and impregnation using an automatic tissue processor (Sakura, Japan). We used an automated embedding station (Sakura, Japan) for embedding the specimens in paraffin blocks. Sections of 4 micron thickness were cut using a rotary microtome (Leica-RM2245, Germany) and then mounted on glass slides. We used an autostainer (Leica Biosystems, Germany) for Hematoxylin & Eosin staining of the sections. The mounted specimens were observed under light microscopy [16, 17].

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used for data evaluation for statistical differences among groups. Dunnett's multiple comparison post-hoc test was used to compare mean values from different treatment groups. P values < 0.05 were considered as statistically significant. All the statistical analysis was performed using the SPSS (Version 10) statistical package.

3. Results

There was no significant difference in serum ALT activities among different treatment groups (Table 2). Serum AST activities showed increasing trends after BAV (0.5 mg/kg) injection and this increase was statistically significant at 24 h following BAV injection (Table 2). The markers of renal function tests including BUN and SCr did not show any significant change among different treatment groups, at different time intervals (Table 2).

The light microscopic observation of liver histopathology is shown in Figure 1. The liver from control rat

Table 1. Sequences of primers used for real time PCR.

Target	Forward primer	Reverse primer
IL-1β	CACCTTCTTTTCCTTCATCTTGT	GTCGTTGCTTGTCTCTCCTTGTA
IL-6	TAGTGGATGCTTCCAAACTG	GAGCATTGGAAGTTGGGGTA
TNF-α	ACTGAACTTCGGGGTGATTG	GCTTGGTGGTTTGVTACGAC
GAPDH	GATTTGGGCGCCTGGTCACC	CGCTCCTGGAAGATGGTGATGG

Table 2. Effects of BAV on biomarkers of liver and renal function test in rats at different time intervals.

Treatment	ALT (U/L)	AST (U/L)	SCr (µmol/L)	BUN (mmol/L)
Control	40.15 ± 3.30	78.44 ± 1.89	21.14 ± 0.85	8.28 ± 0.75
BAV, 2 h	42.45 ± 3.44	91.38 ± 3.52	19.42 ± 0.81	8.54 ± 0.34
BAV, 24 h	42.05 ± 4.86	96.44 ± 6.59*	19.85 ± 1.14	8.18 ± 0.69
BAV, 1 week	48.12 ± 3.56	95.18 ± 12.5	21.57 ± 1.04	7.18 ± 0.27

Values are Mean ± SEM. *P < 0.05 versus control group using Dunnett's test.

showed normal hepatic tissue whereas the injection of BAV (0.5 mg/kg) resulted in congested blood vessels with mild focal inflammatory cell infiltration at 2 h post-dosing (Figure 1B) which persisted at 24 h following BAV exposure (Figure 1C). At one-week post-dosing of BAV, the hepatic cells appeared to be normal indicating the regeneration process with time (Figure 1D).

Figure 2 shows the light microscopic observation of kidney sections from rats of different treatment groups. The control kidney showed normal structures of glomerulus and renal tubules (Figure 2A). Injection of a single dose of BAV (0.5 mg/kg) caused inter-tubular bleeding and inflammatory cell infiltration at 2 h post-dosing (Figure 2B), which gradually subsided at 24 h (Figure 2C) and almost normalized after 1 week (Figure 2D).

There was a significant increase in IL-1β gene expression in liver of BAV (0.5 mg/kg) treated rats as early as 2 h post-dosing, which was further increased at 24 h (Figure 3). However, IL-1β expression reduced with time (1 week) but even significantly higher than control group (Figure 3). There was no significant change in IL-6 gene expression at 2 h post-dosing of BAV, however, significant increases in IL-6 expression were observed at 24 h and 1 week after BAV exposure (Figure 3). Although TNF-α gene expression was not significantly altered at 2 h post-BAV envenomation, a significant increase in TNF-α expression was observed at 24 h that not only persisted but increased after 1 week, post-dosing of BAV (Figure 3).

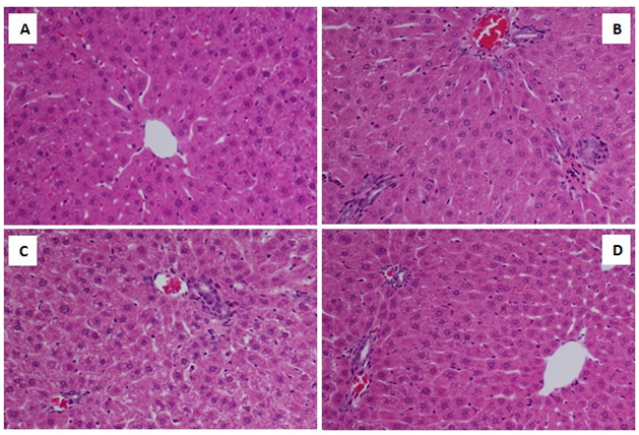

Fig. 1. Light microscopic observation of liver sections of control rat (A) and rats treated with BAV (0.5 mg/kg) and sacrificed after 2 h (B), 6 h (C), 24 h (C) and 1 week (D) (Magnification 200×).

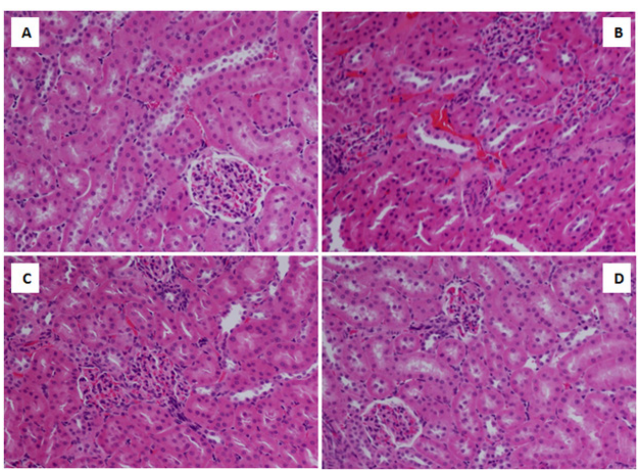

Fig. 2. Light microscopic observation of kidney sections of control rat (A) and rats treated with BAV (0.5mg/kg) and sacrificed after 2 h (B), 6 h (C), 24 h (C) and 1 week (D) (Magnification 200×).

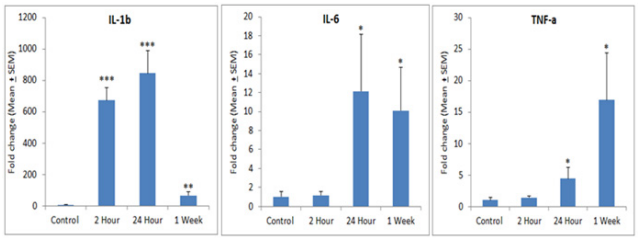

Fig. 3. Effects of BAV on proinflammatory cytokines expression in livers of rats at different time intervals. *P<0.05, **P<0.01 and ***P<0.001 versus control group using Dunnett's multiple comparison test.

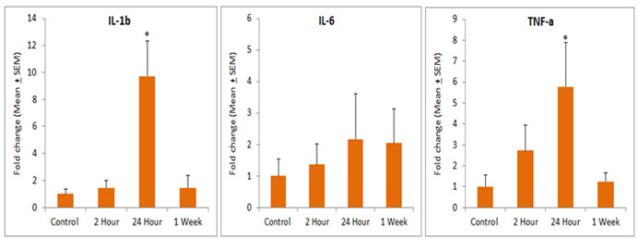

Fig. 4. Effects of BAV on proinflammatory cytokines expression in kidneys of rats at different time intervals. *P<0.05 versus control group using Dunnett's multiple comparison test.

In kidneys, there were no significant alterations on the expression of IL-1β at 2 h and 1 week post-dosing of BAV when compared with the normal rat kidney. However, there was a significant increase in IL-1β gene expression at 24 h after BAV envenomation (Figure 4). The gene expression of IL-6 was not altered by BAV (0.5 mg/kg) at any time points (2 h, 24 h and 1 week) when compared with the control group. BAV envenomation caused significant increase in TNF-α expression at 24 h that subsided after 1 week post-dosing of BAV (Figure 4).

4. Discussion

The results of liver function test did not show any significant change in serum ALT activities among various treatment groups however serum AST was significantly

increased at 24 hours following BAV injection indicating a mild hepatotoxicity (Table 2). Liver histopathology also showed that exposure to BAV (0.5 mg/kg) caused mild focal inflammatory cell infiltration at 2 hours post-dosing which persisted at 24 hours (Figure 1). Although the infiltration of inflammatory cells was also evident in kidneys of rats treated with the sub-lethal dose of BAV (Figure 2), no functional impairment in kidneys was observed in these animals (Table 1). Franca et al [18] observed that exposure to *Crotalus durissus terrificus* snake venom (100 µg/kg) caused inflammatory cell infiltration, endothelial damage, and sinusoidal and portal congestion in rat liver. The hepatic damage caused by inflammatory infiltrate was evident at 3 and 6 hours after venom injection whereas the appearance of tissue returned to normal at 9 and 12 hours post-venom [18]. The envenomation of *Montivipera xanthina* (2.85 mg/kg, intramuscular) in rats resulted in congestion and hepatocellular degeneration in liver sinusoidal cells [19]. *Cerastes cerastes* envenomation of rats caused focal mononuclear white blood cell inflammation, bleeding in sinusoids around the portal area and hepatocyte necrosis tissue in liver [20]. Ali et al [21] reported cell infiltration, inflammation, hepatocyte degeneration, fibrosis, and necrosis in livers of rodents administered with *Hydrophis cyanocintus venom*. On the other hand, exposure to *Bothrops alternatus* snake venom did not show any hepatotoxicity in rats [22].

Al Asmari et al [23] reported that a single IP injection of *Echis pyramidum* (EPV) venom (0.25-1.0 mg/kg) caused rapid impairment in the liver function within 3-6 hours as indicated by significant increases in serum ALT, ALP and GGT activities as well as bilirubin levels. In the same study, the hepatic injury caused by *Echis pyramidum* venom was accompanied with significant and dose-dependent reductions in the activities of antioxidant enzymes (catalase, superoxide dismutase) and glutathione (GSH) together with significant increase in lipid peroxidation in rat liver, suggesting the state of acute oxidative stress within hours following *Echis pyramidum* envenomation [23]. Another study by the same investigators showed that liver and kidney lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) and GSH levels were not affected by the low (0.25 mg/kg) and medium (0.50 mg/kg) doses of EPV, whereas the high dose (1.0 mg/kg) significantly increased TBARS and decreased GSH at 6 hours post-dosing [24]. In mice, the onset of lipid peroxidation was as early as 1 hour and persisted for several hours after intraperitoneal (IP) injection of 2.0 mg/kg dose of EPV [25]. Organ tissue injury due to lipid peroxidation caused by snake venom has been reported both invitro [26] and invivo [25, 27].

The results of real-time PCR showed significant increase in proinflammatory cytokines in liver (Figure 3) and kidneys (Figure 4) of rats injected with BAV. Involvement of the inflammatory cascade and release of proinflammatory cytokines play a crucial role in the pathogenesis of various envenoming syndromes [12]. Macrophage-derived inflammatory cytokines and other cytotoxic mediators are important inducers in pathogenic process [28]. An array of key factors including cytokines, chemokines, interferons, nuclear factor kappa-B (NF-kB), prostaglandins, and oxygen-derived free radicals (ODFR) are responsible for the initiation and propagation of inflammation [29]. A large number of genes, mostly involved in inflammation, apoptosis, ion transport, and energy metabolism have been found to be overexpressed in various organs of mice treated with snake venom; in heart alone, 50% of these genes were differentially expressed [30]. Direct exposure of viper venom to human blood for 2 h showed significant increases in cytoplasmic, lysosomal and extracellular matrix-degrading enzymes as well as pro-inflammatory mediators, indicating a state of acute oxidative stress and inflammation caused by the exposure of viper venom [31]. Yacoub et al [32] reported the production of various proinflammatory cytokines including IL-1β and TNF-α at 6 and 24 hours after exposure of *Montivipera bornmuelleri* (Lebanon viper) venom using peritonitis mice model. *Bothrops atrox* snake venom caused local acute inflammatory reactions, releasing the proinflammatory cytokines TNF-α and IL-6 in mice [33]. Injection of *Bothrops asper* venom in mice showed inflammatory cells in necrotic muscle cells however this pathology was normalized one-week post-envenomation [34].

Inflammatory process is a natural defense mechanism and helps in tissue repair and wound healing. However, uncontrolled inflammation can trigger other pathways of oxidative stress resulting in more tissue damage and loss of function in the inflamed area [35]. It is important to note that intravenous infusion of anti-venoms neutralizes free as well as target-bound toxins but fail to counteract venom-induced inflammation and oxidative stress because the antigen-antibody complex itself acts as pro-inflammatory [31]. However, viper venom-induced cellular damage can be attenuated by antioxidant treatment that inhibits the potentially damaging oxidative cascade and improves membrane stabilization [36-38]. Therefore, a supplementary or adjuvant therapy is important to administer for treating secondary and often overlooked complications of envenomation such as inflammation and oxidative stress. Such kind of adjuvant therapy must be provided at an early stage for more effective protection against acute injury to the organs caused by reactive oxygen species and pro-inflammatory cascade [39].

5. Conclusion

The findings of this study showed that the sub-lethal dose of BAV used in this study did not cause any significant change in liver function except mild increase of serum AST at 24 hours after BAV injection which was also supported by transient structural changes in liver. The biomarkers of renal function including BUN and SCr were not significantly affected by a single dose of BAV (0.5 mg/kg). The histopathology of kidney supported the functional status of kidneys. There were transient increases in proinflammatory cytokines in liver and kidney which tend to reduce with time. These findings suggest the role of inflammation in BAV-induced toxic effects. It is anticipated that higher doses of BAV could result in persistent inflammation which is known to trigger potentially toxic reactive oxygen species that cause cellular damage and organ toxicity.

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Conflict of Interests
The authors have no conflicts with any step of the article preparation.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
The protocol of animal experiments was approved by the Institutional Review Board (Approval No. KSU-SE-21-76). No humans were used in this research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Haseeb A. Khan: Research design, supervision, manuscript writing; Anwar J. Abdulnasir: Literature survey, animal treatment, biochemical analysis; Salman Alamery: Co-supervisoin, laboratory procedures; Nojood A. Altwaijry: Gene expression analysis; Khalid E. Ibrahim: Histopathological analysis.

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