The effect of ascorbic acid on *Bitis arietans* venom induced toxicity in rats

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Abstract Ascorbic acid (AsAc) was tested to evaluate its ability to reverse the oxidative stress induced by envenoming. Test groups of rats were envenomed with sub-lethal doses (4.0 mg/kg s. c.) of *Bitis arietans* venom (BaV) whilst, single doses (500 mg/kg, orally) of AsAc were pre-administered in half of them. Blood samples were collected within three periods and levels of lipid peroxidation (LPO) and total-SH increased significantly, whilst, the 'Venom + AsAc' groups were significantly less than both, the respective 'Venom' groups and controls, at different periods. The antioxidant, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzyme level changes were trivial at the three periods, whilst, there were no changes in the 'Venom + AsAc' groups, compared with controls, except SOD which, became significant after 24 h. SCr and BUN levels were significantly higher than the controls within the three periods with variable degrees, whilst, the 'Venom + AsAc' group level changes were insignificant compared with controls and their respective 'Venom' groups at all periods. Blood urea Nitrogen (BUN), became significantly lower after 24 h. After 6 and 24 h AST levels were significantly higher than controls, whilst, ALT was not. Level changes of both AST and ALT 'Venom + AsAc' groups were insignificant, compared with controls at all periods. It is concluded that oxidative stress due to envenoming by BaV induced variable levels of significant changes in levels of nephrotoxic, hepatotoxic markers and antioxidant enzyme parameters. Administration of AsAc relatively adjusted these changes with different degrees, at variable periods of time that demands further deeper research in beneficiary mechanisms of antioxidants.

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1. Introduction

Snakebites are serious socio-medical problem that lead to morbid and mortal impact on victims, and in Saudi Arabia alone, there are more than 10 terrestrial snake species that are highly venomous (Al-Asmari and Debboun, 2013). The puff adder

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Bitis arietans, and the other vipers have similar serious effects on their victims (Al-Jammaz, 2001), attributed to the complex toxins, enzymes and protein combinations that affect the hepatic, renal, metabolic, cardiovascular and haematological systems, clinically and experimentally (Al-Jammaz, 2001; Fernandez et al., 2014). Prompt antivenom treatment is essential and crucial to avoid poor quality of life and mortality (Lavonas et al., 2002).

The oxidative stress status, which ensues due to snake bite envenoming is another dimension of kidney impairment and acute renal failure (Yamasaki et al., 2008), associated with the antioxidant defence system, that could be subject for countering by antioxidant therapy (Al-Asmari et al., 2014). Reactive oxygen species (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). These free radical ROS, apart from being involved in damaging cellular components, do play a significant role in venom induced toxicity, as had been observed in experimenting with envenomed mice (Dousset et al., 2005). AsAc is an antioxidant that has beneficial effects on several types of cancer (Domitrović, 2006) and could be involved in amelioration of ROS cellular damage, generated during metabolism and exposure to toxins and carcinogens (Banerjee et al., 2009), in addition to enhancement of protease inhibitor effects involved in preventing organ functional damage (Fatani et al., 2006). In this present study, our aim was to investigate the beneficiary effects of AsAc on hepatorenal toxicity and oxidative stress caused by *B. arietans* venom in albino rats.

2. Materials and methods

2.1. Materials (chemicals, reagents and animals)

The reagents, Hydrogen peroxide, Succinic acid, Pyrogallol, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), Tris-base, Potassium phosphate dibasic salt, Potassium phosphate monobasic salt, Hydrochloric acid, Methanol and AsAc were obtained from Sigma, USA. Ready kits for the quantification of serum enzymes (AST and ALT), BUN and Creatinine were obtained from United Diagnostics Industry, Dammam, Saudi Arabia.

B. arietans venom was obtained by manual milking of snakes (both sexes) that were collected from the South Western Regions of Saudi Arabia and maintained in the animal house facility of the Research Center, Prince Sultan Military Medical City (PSMMC), Saudi Arabia. The crude venom was either lyophilized and stored in the fridge at 4 °C or diluted in distilled water and kept frozen in aliquots at -80 °C. Stock solutions of venom were reconstituted in sterile 0.9% NaCl immediately before use.

Male Wistar albino rats, weighing 150–200 g were used in the study. They were obtained and maintained in the animal house facility of the Research Center, PSMMC, Saudi Arabia. They were acclimatized for a week prior to the experiment on standard rat pellet diet with free access to water. Body weights of rats were recorded at the start and completion of the procedure.

2.2. Methods (experimental design and Biochemical analysis)

After acclimatization (7 days) the animals were divided into three different treatment groups (a, b and c) for each test parameter: (a) normal controls (5 rats), (b) venom alone; three groups (according to the injection period of 3 h, 6 h and 24 h) of five rats (15 rats), which were injected with 200 μl/rat venom (4.0 mg/kg s.c.), (c) venom with administration of AsAc; three groups (according to the injection period of 3 h, 6 h and 24 h) of five rats (15 rats), which were injected with 200 μl/rat venom (4.0 mg/kg s.c.). AsAc was administered (500 mg/kg, orally) before venom administration, referring to previous that employed high doses of ascorbic acid (Klenner, 1971; Elshama et al., 2013).

After the completion of treatment schedule and before sacrifice, the rats were anesthetized with anaesthetic ether. Blood was withdrawn from the heart with the help of disposable syringes. Serum was obtained by centrifugation at 3000 rpm for 10 min.

Liver was carefully separated from the treated and control animals, and homogenized according to several current methods (Doss and Anand, 2012) in 0.1 M Tris–HCl buffer pH 7.5 by a glass-Teflon homogenizer (Thomas PA, USA) by passing 5 pulses; at 4 °C to make a 10% w/v homogenate. The homogenate was then subjected to high speed Ultra-Turrex Kunkel homogenizer (Type T-25, Janke & Kunkel GMBH & Co. KG. Staufen). Homogenate was centrifuged at 3000 rpm at 4 °C for 10 min in Beckman J2-M1 (Beckman instruments, Inc Palo Alto, C.A. USA) high-speed refrigerated centrifuge to remove the cell debris. The supernatant was saved in aliquots and stored at -20 °C for assay analyses of enzymatic antioxidant parameters, as described below.

All serum parameters i.e. AST, ALT, BUN and SCr were done using commercially available kits from United Diagnostics Industry, Dammam, K.S.A. Readings were done spectrophotometrically and data were collected in duplicate, according to manufacturer's procedure.

The activities of the three key antioxidant enzymes of the defence system i.e. superoxide dismutase, catalase and glutathione peroxidase were measured simultaneously in the liver homogenate extracts under similar conditions using same solutions to avoid day-to-day experimental variations.

Superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund (1974). To 0.05 ml of supernatant 2.85 ml of 0.05 mM Tris-buffer, pH 8.2 was added, mixed well and incubated at 25 °C for 20 min. The reaction was started by adding 0.1 ml of 8 mM pyrogallol solution. Change in absorbance per minute was immediately recorded for the initial 3 min at 420 nm. A reference set, containing 0.08 ml distilled water instead of supernatant solution, was also run simultaneously.

Catalase (CAT) was assayed according to the method of Claiborne (1985) as described by Giri et al. (1996). The assay mixture consisted of 1.95 ml of 0.05 M potassium phosphate buffer pH 7.0, 1 ml of 0.019 M hydrogen peroxide and 0.05 ml homogenate (50–100 μg protein) in a final volume of 3 ml. The decrease in absorbance at 240 nm was immediately noted after every 30 s for 3 min. Enzyme activity was calculated using the molar extinction coefficient of H_2O_2 (436 M^{-1} cm^{-1} at 240 nm).

The activity of glutathione peroxidase (cGPx) was determined by the method of Flohe and Gunzler (1984). The assay mixture contained 500 μl of 0.1 M potassium phosphate/1 mM EDTA buffer (pH 7.0), 50 μl of 1 mM of sodium azide, 50 μl of homogenate (100–200 μg protein), 100 μl glutathione reductase (0.24 U), and exactly 100 μl of 10 mM GSH. The mixture was preincubated for 10 min at 37 °C. Then 100 μl of 1.5 mM NADPH solution was added and the hydrogen peroxide independent consumption of NADPH was monitored for 3 min. The overall reaction was started by adding 100 μl of pre-warmed 1.5 mM hydrogen peroxide solution and the decrease in absorbance at 340 nm was monitored for 5 min. Enzyme activity was calculated using the molar extinction coefficient of NAPDH ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

The total protein content in liver homogenate was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The liver homogenate was used for the assay of non-enzymatic antioxidant parameters.

Total sulphydryl groups (Total-SH) groups were determined by the method of Sedlak and Lindsay (1968). To 0.4 ml of 10% tissue homogenate, 2.1 ml of 0.1 M Tris–HCl (pH 8.2), 0.5 ml of 10% SDS and 0.3 ml of 0.1 M EDTA were added. The reaction was incubated in a boiling water bath for 5 min. Then, 0.1 ml DTNB (40 mg/100 ml methanol) was added. After 30 min at room temperature, the absorbance was read at 412 nm. A calibration curve with different amounts of cysteine (20–160 nmoles) was constructed by the same procedure as described above and used to calculate the total-SH groups in the samples.

Lipid peroxidation (LPO) in various tissue homogenates was determined spectrophotometrically by the method of Ohkawa et al. (1979). To 0.1 ml of tissue homogenate 1.5 ml of 20% trichloroacetic acid, 0.2 ml of 8% SDS, 0.7 ml of distilled water and 1.5 ml of 0.8% thiobarbituric acid were added and after mixing incubated at 95 °C for 20 min. After cooling to room temperature and centrifugation at 10,000 rpm for 10 min the absorbance of the supernatant was read at 532 nm against reagent blank. Amount of malondialdehyde (MDA, end product of LPO) was calculated using the molar extinction coefficient of thiobarbituric acid ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}).

2.3. Statistical analysis

Graphpad Instat and Graphpad Prism 3.11 program software were used. Data were analyzed by a one way analysis of variance (ANOVA) with Bonferroni's post multiple comparison tests. A two way analysis of variance was also done to compare in between groups. $P \leqslant 0.05$ was considered significant.

3. Results

With respect to stress parameters, administration of BaV in rats led to increased levels of LPO and total-SH with respect to controls and highly significant levels were seen after 6 h ($^{***}p < 0.001$) and 24 h ($^{***}p < 0.001$) in case of LPO, whilst, it was highly ($^{***}p < 0.001$) significant within 6 h in case of total-SH (Table 1).

On the other hand, administration of venom with AsAc ('Venom + AsAc' group) showed an insignificantly lower level of LPO (MDA) than the 'Venom' and 'Control' groups at 3 h and 6 h but, at 24 h differences were significantly lower than the respective 'Venom' group, whereas, total-SH levels of the 'Venom + AsAc' were only insignificantly lower than its respective 'Venom' group at 6 h and was also insignificantly lower than the controls after 24 h (Table 1).

With respect to the antioxidant enzyme parameters of the 'Venom' groups, SOD levels were insignificantly lower at 3 h but, they increased insignificantly after that. CAT level was insignificantly lower than controls but, increased insignificantly only after 24 h. Levels of GPx were lower within the three periods with respect to controls but, significance ($^{*}p < 0.05$) was only seen at the beginning of 3 h test, (Table 1).

On the other hand, venom and AsAc in 'Venom + AsAc' groups, showed that SOD levels were insignificantly higher than the 'Control' group at 3 h and 6 h but, increased significantly ($^{*}p < 0.05$) after 24 h. CAT levels of the 'Venom + AsAc' group were significantly ($^{*aa}p < 0.05$ and $^{**a}p < 0.01$) higher after 3 h and 24 h, respectively, with the respective 'Venom' groups and the controls. With respect to GPx, the 'Venom + AsAc' groups had insignificantly ($p > 0.05$) higher levels at 3 h, 6 h and 24 h than the respective 'Venom' group and controls (Table 1).

With respect to the nephrotoxic non-enzyme parameters of the 'Venom' groups, SCr levels were significantly ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) higher than the controls after 3 h, 6 h and 24 h, respectively. Whilst, BUN levels were insignificantly higher at the first period but, they increased significantly ($^{**}p < 0.01$ and $^{***}p < 0.001$) after that (6 h and 24 h), respectively (Table 2).

Table 1 Effect of *Bitis arietans* crude venom on enzymatic/non-enzymatic levels in rat liver homogenate.

Group	LPO (nM/g)	Total-SH (μM/g)	SOD (μM/min/mg)	CAT (μM/min/mg)	GPx (μM/min/mg)
Control	59.21 ± 1.54	22.19 ± 0.88	7.89 ± 0.28	34.42 ± 2.83	0.35 ± 0.07
Venom 3h	60.59 ± 1.80	20.63 ± 0.35	7.78 ± 0.32	24.49 ± 2.17	0.08 ± 0.02*
Venom + AsAc 3h	57.78 ± 1.82	22.68 ± 0.71	8.01 ± 0.40	61.71 ± 7.70^{*aa}	0.40 ± 0.10^{a}
Venom 6h	88.29 ± 3.51***	28.61 ± 0.39***	8.43 ± 0.40	33.58 ± 4.40	0.11 ± 0.04
Venom + AsAc 6h	77.22 ± 3.90	27.00 ± 0.27	8.29 ± 0.30	39.08 ± 3.94	0.26 ± 0.03
Venom 24h	109.71 ± 3.53***	20.34 ± 0.60	9.02 ± 0.61	38.89 ± 3.39	0.25 ± 0.04
Venom + AsAc 24h	85.69 ± 4.35^{*aa}	20.78 ± 0.52	9.58 ± 0.25*	85.92 ± 6.92^{**a}	0.35 ± 0.05

Data were analyzed by a one way analysis of variance (ANOVA) with Bonferroni posts multiple comparison tests. A two way analysis of variance was also done to compare in between groups. $P \leqslant 0.05$ was considered significant. Values are Mean ± SEM.
$^{*}p < 0.05$ as compared to control; $^{a}p < 0.05$ as compared to the respective group; $^{**}p < 0.01$ as compared to control; $^{aa}p < 0.01$ as compared to the respective group; $^{***}p < 0.001$ as compared to control; $^{aaa}p < 0.001$ as compared to the respective group.
^{*aa} = This group is $^{*}p < 0.05$ as compared to control, and also it is $^{aa}p < 0.01$ as compared to the respective group (its venom alone group).

Table 2 Effect of *Bitis arietans* crude venom on serum parameters.

Group	Creatinine (mg/dl)	BUN (mg/dl)	AST (U/L)	ALT (U/L)
Control	1.03 ± 0.06	23.78 ± 0.80	75.90 ± 3.90	35.78 ± 1.49
Venom 3h	$1.25 \pm 0.05^*$	25.72 ± 0.80	76.58 ± 1.70	$25.40 \pm 1.26^*$
Venom + AsAc 3h	1.22 ± 0.02	23.78 ± 0.60	80.29 ± 3.40	27.48 ± 3.54
Venom 6h	$1.31 \pm 0.06^{**}$	$29.63 \pm 1.00^{**}$	$102.51 \pm 2.90^*$	34.52 ± 1.12
Venom + AsAc 6h	1.24 ± 0.05	25.00 ± 0.82	91.11 ± 0.07	34.29 ± 1.72
Venom 24h	$1.60 \pm 0.03^{***}$	$33.41 \pm 1.60^{***}$	$141.68 \pm 10.71^{***}$	33.41 ± 1.98
Venom + AsAc 24h	$1.49 \pm 0.04^{***}$	21.62 ± 0.54^{aaa}	97.78 ± 8.40	31.60 ± 2.60

Data were analyzed by a one way analysis of variance (ANOVA) with Bonferroni posts multiple comparison tests. A two way analysis of variance was also done to compare in between groups. $P \leqslant 0.05$ was considered significant. Values are Mean $\pm$ SEM.
[*] $p < 0.05$ as compared to control; [a] $p < 0.05$ as compared to the respective group; [**] $p < 0.01$ as compared to control; [aa] $p < 0.01$ as compared to the respective group; [***] $p < 0.001$ as compared to control; [aaa] $p < 0.001$ as compared to the respective group.

On the other hand, venom and AsAc in 'Venom + AsAc' groups, showed that SCr levels were insignificantly higher than controls at all periods but, was significant ([*] $p < 0.05$) after 24 h, though, they were insignificantly ($p < 0.05$) lower than the respective 'Venom' groups at all periods. With respect to BUN, the 'Venom + AsAc' group levels were insignificantly ($p > 0.05$) higher at 3 h and 6 h but, after 24 h they were insignificantly ($p > 0.05$) lower than controls. Compared with the respective 'Venom' groups, levels were insignificantly ($p > 0.05$) lower at all periods but, after 24 h the difference became significantly ([aaa] $p < 0.001$) lower (Table 2).

With respect to the hepatotoxic enzyme parameters, the 'Venom' group levels of, AST were insignificantly lower at the beginning of 3 h and but, after that (6 h and 24 h), they were significantly ([*] $p < 0.05$ and [***] $p < 0.001$) higher, respectively. Levels of ALT were insignificantly ($p > 0.05$) lower than controls at all periods but, significantly ([*] $p < 0.05$) lower at the 3 h start period (Table 2).

On the other hand, venom and AsAc in 'Venom + AsAc' groups, showed that AST levels were insignificantly ($p > 0.05$) higher than the 'Control' group at all periods but, were also insignificantly ($p > 0.05$) higher than the respective 'Venom' groups only within 3 h period. ALT levels of the 'Venom + AsAc' groups were insignificantly ($p > 0.05$) lower than the 'Control' group at all periods but, were insignificantly ($p > 0.05$) higher than the respective 'Venom' group only within 3 h period (Table 2).

4. Discussion

Antivenom is the treatment of choice following envenoming by snakes, especially the viper *B. arietans* (Dey and De, 2012) and the components of their venoms lead to serious toxic effects and alterations on their victims and experimental rat metabolism (Al-Jammaz, 2001). Results of this recent study, of liver tissue LPO (MDA levels) showed increased levels that were highly significant, which indicated hepatic injury. Other works had reported this (Otuechere et al., 2014) whilst lowered levels indicated hepatoprotective activity (Arthur et al., 2012).

The use of some herbal hepatoprotective antioxidant extracts had significantly lowered high levels of MDA (Dey and De, 2012, 2011). Significantly high levels of total-SH (within 6 h) and LPO levels apparently refer to the envenoming factors of the viper venom, whilst the administration of AsAc (AsAc) significantly decreased LPO and total-SH levels at vari-

able periods. In other reports AsAc effectively provided protection against both, of low density lipoprotein and lipids (Chen et al., 2000), in addition to other works on human plasma (Chen et al., 2000; Berger et al., 1997), previous works on rats, resisting viper venoms (Fatani et al., 2006; Arthur et al., 2012) and diabetic rabbits (Ojieh et al., 2009).

This study showed that SOD, CAT and GPx (the enzymatic antioxidants) have undergone exhaustion, following BaV administration but, their levels were significantly increased with AsAc administration. The significant differences of these antioxidant enzymes reflected the beneficial and protective ability of AsAc. Other extracts of hepatoprotective antioxidant plants and vitamin E were found to behave accordingly (Gumieniczek et al., 2002) whilst, Cu-Zn SOD was reported to have significant similar activity in conditions of diabetic and cardiac case diseases (Gumieniczek et al., 2002; Iwueke et al., 2001).

This situation necessitated the trends initiated to isolate, purify and characterize the active biomolecular extracts in plants that were used as effective inhibitors in reducing the local tissue damage and toxicity of snake venoms (Chatterjee et al., 2006). Variability in treatment put antivenom as priority, being the only available treatment for snake bite (Gomes et al., 2010) with its multiple and complicated several limitations (Gomes et al., 2010; Datta et al., 2011). Parameters of nephrotoxicity SCr and BUN were significantly high after venom administration, reflecting envenoming stress, as had been reported previously (Valenta et al., 2010). The high intoxication level of the nephritic system due to viper envenoming was also reported in clinically envenomed cases (Valenta et al., 2010; Sagheb et al., 2011).

AsAc also showed a significantly beneficial effect on the fate of elevated nephrotoxic parameters, similar to what happened in case of LPO and total-SH, and BUN levels were even lower than the control itself, after 24 h. Previous similar studies also resulted in alleviation and reversal of critical situations, back to normal (Natural Paws, 2007). Renal failure following snake bites has a multifactorial pathogenesis (Valenta et al., 2010), reflecting renal function deterioration that becomes apparent within hours, days or even weeks (Valenta et al., 2010).

As a target of snake venoms, liver injury will release AST and ALT in serum levels, which were highly significant, according to the present study. Restoration of these levels to normal, following AsAc administration is indicative of some protection degree, reported by previous studies (Natural Paws, 2007).

Tissue injury usually leads to anaphylaxis and onset of inflammation due to snake venom PLA_2 that aids and enhances tissue disruption. Supportive to this but, in a serious way is the generation and production of tissue destructive free oxygen radicals that cause highly reactive lipid peroxides which are real markers of viperine and crotaline envenoming, added to alterations and inflammatory responses, both in humans and experimental animals at the injection sites (Teixeira et al., 2009). Similar effects of lethal venom doses had been reported earlier (Rahmy and Hemmaid, 2001). Furthermore, the antioxidant herbal extracts that contain flavonoids were thought to reverse hepatotoxicity and nephrotoxicity by depleting the pro-oxidants generated during metabolism of toxic oxidative agents (Arthur et al., 2012; Myhstad et al., 2002) and probably viper venoms.

Then, AsAc ameliorating effects had more than startling powers in improving all sick and affected systems at the microcellular levels which is reflected in antagonizing snake venom components that induce anaphylaxis or at least alleviating their drastic allergenic effects, as Lee (2000) reported that AsAc could prevent allergies by detoxifying allergens before triggering their reactions. Treatment of snake bite solely based on the use of AsAc was started and practiced by Dr. Klenner (1971) who saved some envenomed lives bitten by dangerous snakes, by administering high doses of AsAc (i.v.). The use of mega doses of AsAc showed that it acted as a reducing agent, an oxidizing agent, an anti-clotting agent, an antihistamine, and as an anti-infective agent (Vitamin C Foundation, 2004; Elshama et al., 2013). This is to reflect some aspects of AsAc prophylactic activities, in line with reflections on alternative food and plant extract treatment coupled with the current employment of antivenom serotherapy.

5. Conclusions

It is concluded that administration of AsAc successfully kept the levels of the antioxidant enzymes and other biochemical blood parameters of the hepatic and nephritic systems of rats injected with sub-lethal doses of viper (BaV) snake venoms within the limits of normal levels. These findings suggest that large doses of administered AsAc coupled with available antivenoms, could be effective in bringing about healing effects and functional improvement of hepatocytes and nephrocytes. This may pave the way for further and deeper research, bearing the information that AsAc might have a potential therapeutic approach to counter viper snake envenoming properties.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

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