Cardiac disorders and mode of action of the Egyptian scorpion venom *Androctonus bicolor* on isolated toad’s heart

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Cardiac disorders; ECG; Ion channel blockers; Scorpion toxins; Scorpion envenomation

**Abstract**
Scorpion venom is a complex mixture of components with various pharmacological and toxicological effects. It is characterized by the presence of a large number of toxins that specifically interact with ion channels of excitable cells. The Egyptian scorpion *Androctonus bicolor* belongs to the family of Buthidae and until now no information is available about the effect of its venom on cardiac muscles. Using an *in vitro* approach, cardiotoxicity and mode of action of *A. bicolor* venom on isolated toad’s heart were investigated. Direct application of scorpion venom (0.5 µg/ml) into isolated toad’s heart induced a remarkable bradycardia concomitant with a protraction in the conduction time (P–R interval). In the meantime, a significant increase in the R-wave amplitude (ventricular contraction) was noticed after 5 min of venom perfusion. Various cases of cardiac disorders were recorded such as sinus arrhythmias, ectopic beats and different degrees of heart block. Through using different autonomic and ion channel blockers, the possible mechanism of action of *A. bicolor* venom on isolated toad’s heart was revealed. The application of both atropine (4 µg/ml) and verapamil (5 µg/ml) could not alleviate the pronounced negative chronotropic and positive inotropic effects. Meanwhile, a significant decrease in the R-wave amplitude (ventricular contraction) was noticed after 5 min of venom perfusion. Various cases of cardiac disorders were recorded such as sinus arrhythmias, ectopic beats and different degrees of heart block. Through using different autonomic and ion channel blockers, the possible mechanism of action of *A. bicolor* venom on isolated toad’s heart was revealed. The application of both atropine (4 µg/ml) and verapamil (5 µg/ml) could not alleviate the pronounced negative chronotropic and positive inotropic effects. Meanwhile, a significant decrease in the R-wave amplitude (ventricular contraction) was observed after propranolol (5 µg/ml) application. In conclusion, our findings indicate that the venom of *A. bicolor* directly influenced the cardiac electrical activity of toads through β-adrenergic receptors. The direct effect of this venom on cardiac tissues may significantly contribute in the development of several cardiotoxic effects following scorpion sting.

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**Introduction**
Scorpions are venomous arthropods and one of the oldest arachnids (<400 million years ago). They are a modest group
containing 1500 different species in 16 families (Fet et al., 2000). Of these, the family of Buthidae is the largest (80 genera and over 800 species) and the most medically important (Soleglad and Fet, 2003). Scorpion venom is a heterogeneous mixture of substances including nucleotides, biogenic amines, lipids, peptides and proteins. Recent venomics studies revealed that each scorpion species may contain more than 150 toxins and venom peptides (Rodríguez de la Vega and Possani, 2005; Abdel-Rahman et al., 2013, 2014). Peptides with biological properties can be classified into non-disulfide-bridged peptides (NDBPs) and disulfide-bridged peptides (DBPs) (Possani et al., 1999; Zeng et al., 2005). However NDBPs are a small group, they exhibit various toxicological and pharmacological properties including hemolytic, anticancer, antimicrobial, anti-inflammatory, anti-epileptic, immune-modulatory and bradykinin potentiating activities (see a review, Almala et al., 2014; Harrison et al., 2014). On the other hand, DBPs represent the major scorpion venom constituents (neurotoxins) responsible for the neurotoxic and cardiotoxic effects induced during envenomation. Until now there are more than 250 neurotoxins that have been identified from the venom of various scorpion species (Abdel-Rahman et al., 2014, 2015). Specifically, these peptides (23–76 amino acids stabilized by three or four disulfide bridges) modulate various types of mammalian and insect ion channels such as Na+, K+, Ca2+ and  

rotoxins that have been identified from the venom of various areas (Chippaux and Goyffon, 2008). The approximate number of scorpion stings is greater than one million per year, resulting in massive cases of morbidity and mortality (Sofer et al., 2013; Coelho et al., 2014). According to several epidemiological and clinical studies (e.g., Abroug et al., 1993; Elatrous et al., 1999; Ribeiro et al., 2010; Bahloul et al., 2013; Sofer et al., 2013), the severity of scorpion envenomation was divided into three main levels based on clinical signs and symptoms. The first level includes victims with only local pain at the site of scorpion sting; the second level includes patients with systemic manifestations and the third level includes patients with cardio-respiratory disorders (Bouaziz et al., 2008). Congestive heart failure, pulmonary edema, arterial hypertension and cardiac muscular lesions are the most severe cardiovascular and haemodynamic alterations (Cupo et al., 2007). Myocarditis induced after scorpion envenomation might be caused by the direct effect of venom on cardiac muscles (Teixeira et al., 2001), or through indirect pathways (releasing of catecholamines, accumulation of lactic acids and vasoconstriction) (Murthy and Hase, 1994; Omran et al., 1994; Ismail, 1995; Meki et al., 2003). Moreover, cardiac abnormalities (sinus tachycardia and bradycardia, sinoatrial and atrioventricular block, ventricular ectopic beats and idioventricular rhythm) have been recorded after the application of scorpion venoms (e.g., Leirius quinquestratius quinquestratius, Mesobuthus tumultus, Tityus serrulatus) on experimental animals (Omran et al., 1992a, 1994; Teixeira et al., 2001).

Several recent technological approaches (e.g., proteomics and transcriptomics) are being used to study venomics of different scorpions. These studies are mainly focusing on the separation and identification of novel venom molecules and reveal their mode of action. At the same time, classical studies are urgently needed to draw an overview about toxic effects of scorpion crude venoms. In Egypt, there are four different scorpion species belonging to the dangerous genera of Androctonus (Androctonus australis, Androctonus crassicauda, Androctonus amoreuxi, and Androctonus bicolor). There are various physiopathological and pharmacological studies about the species of A. australis, A. crassicauda and A. amoreuxi (e.g., Saidi et al., 2013; Aghabikloo et al., 2013; Fetaih et al., 2013). However, no information is available about the black fat-tailed scorpion or A. bicolor. In this study, direct cardiotoxic effects of the Egyptian scorpion A. bicolor venom were evaluated using isolated toad’s heart. Additionally, the possible mode of action of this venom on cardiac muscle was revealed.

Materials and methods

Ringer solution and blockers

Physiological Ringer solution (6.5 g/L NaCl, 0.14 g/L KCl, 0.12 g/L CaCl2, 0.2 g/L NaHCO3, 0.01 g/L NaH2PO4 and 1.0 g/L Glucose) was used (4 μg/ml) in all heart experiments and blocker preparations (Chapman et al., 1979; Nabil et al., 1998; Abdel-Rahman et al., 2010). The blockers of atripine sulfate 0.1% (muscarnic acetylcholine receptor blocker, Memphis Co., Cairo, Egypt), verapamil hydrochloride 40 mg (calcium channel antagonist, EL Nasr Pharm. Chem. Co., Abu Zirbal, Egypt) and propranolol hydrochloride 40 mg (β-adrenergic receptor blocker, AstraZeneca Co., Egypt) were directly applied on toad’s hearts with final concentrations of 4, 5 and 5 μg/ml, respectively.

Scorpions and venom collection

Adult scorpion specimens were collected from the Western Mediterranean Costal Desert (Alexandria Governorate, Egypt). They were kept alive in individual containers containing sand, with controlled temperature and humidity. They were fed on cockroaches and received water ad libitum every two weeks. The venom was obtained using electrical stimulation (16 V, 5–10 s). The milked venom was collected in Eppendorf tubes and solubilized in bi-distilled water. The soluble venom was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was pooled; freeze dried (Labconco Freeze Dry System, Kansas, USA) and the stock was stored at −20 °C until use (Abdel-Rahman et al., 2013). The venom working solution (0.5 μg/ml) used in all heart experiments was freshly prepared in Ringer solution.

Experimental animals and isolation of toad’s hearts

The Egyptian adult male toads of Bufo regularis (35–40 g) were used in this study. All experiments were conducted in accordance to the guide for the care and use of laboratory animals. The toads were kept (19–22 °C) in the animal house of Zoology Department with free access to water and were fed mealworms once a week. To isolate toad’s hearts, adult male toads were pithed with a fine stainless steel needle and placed...
ventral side up in a wax dissecting pan. Fine forceps were used to grasp the skin over the center of pectoral girdle and sharp scissors were used to cut the skin. The xiphoid cartilage was raised by forceps and kept high to prevent heart damage. The muscle layer and the pectoral girdle were cut and then the covering pericardium was removed to expose the heart. The dissecting heart was kept moist throughout the investigation by pipetting room temperature Ringer’s solution over the preparation. Carefully the heart was isolated by a scissors and placed in Ringer’s solution to remove the blood.

ECG recording and experimental design

Electrocardiogram (ECG) was recorded directly from the surface of the toad’s heart according to Nabil et al. (1998). Based on our preliminary experiments of testing different concentrations, 0.5 μg/ml of the scorpion venom was chosen and directly applied into the heart. ECG was taken before any treatment to act as self-control (0 min). After venom perfusion into the heart, signals were recorded at different time intervals of 5, 10, 15, 20, 25 and 30 min. In order to allow free movement of the isolated heart, two pieces of cotton threads (5 cm each) were immersed in Ringer’s solution. The end of one wet thread closely touched the right atrium and the second the apex of the ventricle. ECG signals were picked up through two silver wires connected with the cotton threads, magnified and displayed by the multi-pen rectilinear recorder (DBE, UK) with variable paper speeds (2 and 10 mm/s). To explore the possible mode of action of A. bicolor venom, various antagonists (atropine sulfate, verapamil and propranolol) were applied after the multi-pen rectilinear recorder (DBE, UK) with variable paper speeds (2 and 10 mm/s). To explore the possible mode of action of A. bicolor venom, various antagonists (atropine sulfate, verapamil and propranolol) were applied after 5 min (maximum effect of scorpion venom was recorded at this time) of venom perfusion.

Statistical analysis

Data were statistically analyzed using SPSS v. 17.01 (SPSS Inc., Illinois, USA) and presented as mean ± standard error (SE). The analysis of a one-way ANOVA was applied to assess significant differences (P < 0.05) in the heart rate (HR) and different ECG measurements between control and treated groups. The effects of different antagonists (atropine, propranolol and verapamil) on isolated toad’s hearts were evaluated using Student’s paired t-test (P < 0.05).

Results

Effect of A. bicolor venom on the electrical activity of toad’s hearts

Various parameters related to the electrical activity of the heart were measured before (self-control) and after the direct application of scorpion venom (0.5 μg/ml). The recorded ECG parameters include heart rate (HR), conduction velocity (P–R interval), voltage of depolarization (R-wave) and repolarization (T-wave) (Table 1).

The A. bicolor venom induced remarkable bradycardia (decrease in HR) in the isolated toad hearts. This pronounced negative chronotropic effect started after 10 min (51.44 ± 4.298 beats/min) from venom perfusion and peaked at 30 min (37.07 ± 3.63 beats/min). Moreover, a highly significant difference was detected (P < 0.001) in the HR between the self-control and the treated groups (5, 10, 15, 20, 25 and 30 min) using a one-way ANOVA (Table 1; Fig. 1A). Interestingly, the negative chronotropic effect of scorpion venom was concomitant with a noticeable negative dromotropic effect (prolongation of P–R interval). The data in Table 1 and Fig. 1B showed that the venom of A. bicolor significantly decreased the conduction velocity of impulses during the whole course of the experiment and the maximum prolongation of P–R interval was recorded at 30 min (745 ± 88.40 ms).

Scorpion venom of A. bicolor caused a positive inotropic effect indicated by a prominent increase in myocardial contractility (R-wave amplitude). Venom inotropic effect was quickly evident and significantly increased at 5, 10 and 15 min (Table 1, Fig. 1C). Similarly, the ventricular repolarization (T-wave amplitude) was significantly elevated from the onset of venom application (5 min) and it sustained until 30 min. As shown in Fig. 1D, the highest value of T-wave was recorded after 10 min from venom application. Using a one-way ANOVA, we detected significant differences in the amplitude of both R and T-waves between the control (0 min) and the treated groups (5–30 min).

ECG abnormalities

ECG charts of perfused toad’s hearts (n = 10) with 0.5 μg/ml of A. bicolor venom are illustrated in Fig. 2. The venom

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>HR (beats/min)</th>
<th>P–R interval (ms)</th>
<th>R-amplitude (mV)</th>
<th>T-amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>61.41 ± 3.043</td>
<td>340 ± 18.753</td>
<td>1.75 ± 0.104</td>
<td>0.38 ± 0.071</td>
</tr>
<tr>
<td>5</td>
<td>59.01 ± 1.868</td>
<td>440 ± 23.169</td>
<td>2.9 ± 0.26</td>
<td>1.14 ± 0.24</td>
</tr>
<tr>
<td>10</td>
<td>51.44 ± 4.298</td>
<td>515 ± 30.498</td>
<td>2.72 ± 0.225</td>
<td>1.72 ± 0.214</td>
</tr>
<tr>
<td>15</td>
<td>51.3 ± 4.077</td>
<td>560 ± 39.432</td>
<td>2.41 ± 0.299</td>
<td>1.52 ± 0.200</td>
</tr>
<tr>
<td>20</td>
<td>46.95 ± 4.155</td>
<td>630 ± 51.986</td>
<td>2.2 ± 0.320</td>
<td>1.38 ± 0.205</td>
</tr>
<tr>
<td>25</td>
<td>43.52 ± 5.139</td>
<td>705 ± 93.990</td>
<td>1.96 ± 0.287</td>
<td>1.22 ± 0.182</td>
</tr>
<tr>
<td>30</td>
<td>37.07 ± 3.626</td>
<td>745 ± 88.409</td>
<td>1.84 ± 0.245</td>
<td>1.12 ± 0.172</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SE (n = 10/group).

* Significant different (P < 0.05) from control group (0-time) using Student’s paired t-test.

** Significant difference (P < 0.05) between control (0-time) and treated groups (5, 10, 15, 20, 25 and 30 min) using a one-way ANOVA.
application induced bradycardia, P–R prolongation, elevation in the voltage of ventricular depolarization and repolarization. Also, various cases of ECG abnormalities related to rhythmicity (Fig. 3, Plate I) and conductivity (Fig. 3, Plate II) of the heart were recorded. The rhythmicity cases (Plate I) were sinus arrhythmia (trace B), bradycardia (trace C), ventricular ectopic beats (trace D), widening of the QRS complex (trace E), ventricular fibrillation (trace G), ischemia (trace D and E) and infraction (trace F). On the other hand, conductivity cases (Plate II) were represented by several degrees of heart blocks; first degree heart block (trace B), second degree heart block (trace C) and complete heart block (trace D).

Mechanism of action

The possible cardiotoxic mechanism of action of this venom was examined through the application of different autonomic and ion channel blockers (atropine sulfate, verapamil and propranolol; Fig. 4). In order to abolish the bradycardia and the decreased atrioventricular conduction velocity induced by the scorpion venom, atropine sulfate (muscarinic receptors antagonist) was perfused (4 µg/ml) post venom treatment. The application of atropine sulfate could not attenuate the negative chronotropism and dromotropism elicited by A. bicolor venom (Fig. 4A and B). In an attempt to explain the remarkable increase in cardiac muscle contractility, verapamil (selective L-type Ca2+ channels antagonist) and propranolol (β-adrenergic receptors antagonist) were used after venom application. The data in Fig. 4(C and D) showed that application of verapamil (5 µg/ml) could not inhibit the positive inotropic effect of scorpion venom. On the hand, positive inotropic effect of scorpion venom was abolished after the addition of propranolol (5 µg/ml). We noticed that the significant (P < 0.05) increase in myocardial contractility after 10 min of venom application returned to the control value after propranolol perfusion (Fig. 4E and F).

Discussion

Scorpion venom contains various types of neurotoxins modulating ion channels of excitable cells. Using an in vitro approach (isolated toad’s heart), the present study was carried out to investigate cardiotoxic effects and the mechanism of action of A. bicolor venom. Direct application of scorpion venom (0.5 µg/ml) on isolated toad’s heart induced a significant negative chronotropic effect (bradycardia). The resulted bradycardia appeared from the onset and stand till the end of experiment. It was reported that injection of the Buthidae scorpion venom Buthus occitanus into rabbits caused an immediate short lasting bradycardia followed by tachycardia then a prolonged bradycardia (Ismail et al., 1980). On the other hand, Teixeira et al. (2001) found that Tityus serrulatus scorpion venom applied on isolated rat’s heart caused an initial decrease followed by an increase in heart rate. Moreover, injection of small doses from the Egyptian scorpion venom L. quinquestriatus into anesthetized rats induced sinus tachycardia while high
doses produced sinus bradycardia (Omran et al., 1992a,b, 1994). The authors explained the induction of bradycardia recorded after administration of high dose of the venom as a reflex action, to overcome the positive chronotropism and inotropic features by increase the vagal withdrawal or release. These differences in the data could be attributed to different mechanisms of toxin action of scorpion species.

The pronounced bradycardia caused by application of $A.\ bicolor$ venom was not abolished by atropine (muscarinic cholinergic antagonist). This result elucidates that the bradycardia caused by the venom may not have a cholinergic basis. So, the neurotransmitter-independent decrease in heart rate appears to be a direct effect of the venom on the myocardium. The same results were obtained by Omran et al. (1992b) with the venom of $L.\ quinquestriatus$. They found that application of atropine before the injection of $L.\ quinquestriatus$ venom has no role to prevent bradycardia induced by scorpion venom. In contrast, Ismail et al. (1980) and Teixeira et al. (2001) reported the ability of atropine to reverse the bradycardia produced by the scorpion venoms of $B.\ occitamus$ and $T.\ serrulata$ which reflecting parasympathetic activity of scorpion venom. In the present investigation, the negative chronotropic effect of $A.\ bicolor$ venom was concomitant with a significant negative dromotropic effect (prolongation in the atrioventricular conduction or P–R interval). This effect could be related to the depression of atrioventricular conductivity in treated hearts (van den Berg et al., 1994; Abdel-Rahman et al., 2010). A different possible mechanism could involve the ATP-sensitive potassium ($K_{ATP}$) channels, also termed the ADP-activated potassium channel, which is a ligand-gated channel distributed abundantly in all regions of the heart (Seino and Miki, 2003). The open probability of this channel is proportional to the $[\text{ADP}]/[\text{ATP}]$ ratio. This channel couples the shape of the action potential to the metabolic state of the cell. Energy depletion during ischemia caused by the venom application could increase the $[\text{ADP}]/[\text{ATP}]$ ratio, thus activating $K_{ATP}$, and abbreviating the action potential. The abbreviated action potential may be cardioprotective.

However, it does not seem that the protective effects of the activation of ATP-sensitive potassium channels are able to counteract the deleterious effects of $A.\ bicolor$ venom since it has a cardiac stimulatory effect illustrated in the positive inotropism (increase in ventricular contractile force or $R$-amplitude, Fig. 1) shown upon the venom application. Several pathways may be responsible for the noticeable inotropic effect of $A.\ bicolor$ venom: (i) the ability of scorpion venom to release catecholamines from nerve terminals leading to the activation of $\beta$-adrenergic receptors of heart (Ismail et al., 1980; Omran et al., 1994; Teixeira et al., 2001). We tested this pathway through the post-treatment of propranolol ($\beta$-adrenergic antagonist) which significantly modified the increase of myocardium contraction caused by $A.\ bicolor$ venom.

![Figure 2](image.png)

**Figure 2** Effect of $A.\ bicolor$ venom (0.5 $\mu$g/ml) on the electrocardiogram (ECG) of isolated toad’s hearts. Trace A, before treatment (0 min); traces B, C, D, E, F and G are 5, 10, 15, 20, 25 and 30 min post venom application, respectively.
It is well known that scorpion venom activates sodium, potassium and calcium channels of excitable cells to enhance releasing of catecholamines (Clot-Faybesse et al., 2000). Catecholamines may induce a toxic effect on the cardiac muscle through generation of free radical, enhanced lipid mobility, increased sarcolemmal permeability, calcium overload and alteration in the autonomic tone (Richard and David Hillis, 2008); (ii) inhibition of myocardium Na,K-ATPase by certain toxins of *A. bicolor* followed by an intracellular calcium overload causing strong ventricular contraction (ventricular fibrillation noticed in Fig. 3, Plate 1G supports this opinion). It is worth noting that the inhibition of Na,K-ATPase has been confirmed after the *in vivo* application of venoms from the Buthidae scorpions *T. serrulatus* and *Buthus tumulus* (Krishna Murthy, 1982; Comellas et al., 2003) and (iii) direct effect of *A. bicolor* toxins via the activation of sarcoplasmic reticulum (SR) ryanodine receptors (RyRs) which causes SR calcium release accompanied with strong contraction of heart muscles (Couraud et al., 1980; Valdivia et al., 1992; El-Hayek et al., 1995; Neto et al., 2012). Because the application of verapamil did not mitigate the positive inotropic effect (Fig. 4C and D), it can be proposed that L-type calcium channels are not engaged in the stimulatory effect of *A. bicolor*. The same finding was obtained *in vivo* by Al-Shanawani et al., 2005 who found that verapamil could not protect the mice injected with the venom of *L. quinquestriatus*.

The *in vitro* application of *A. bicolor* induced various cases of cardiac disorders including sinus arrhythmia, ventricular ectopic beats, widening of the QRS complex, ischemia, infraction, ST-T segment changes, ventricular fibrillation and different degrees of heart block (Fig 3, Plates I and II). Several previous studies reported different cardiac abnormalities (sinus tachycardia and bradycardia, atrial fibrillation, AV dissociation with accelerated junctional rhythm, premature atrial or ventricular beats, ventricular tachycardia and ventricular fibrillation, different degrees of heart block) following scorpion envenomation, especially from the family of Buthidae (Gueron et al., 1967; Gueron and Yarom, 1970; Bawaskar and Bawaskar, 1989; Omran et al., 1994; Pinto et al., 2010; Sofer et al., 2013). It was reported that sinus arrhythmia may take place because the sinus node does not discharge with absolute regularity due to alteration in vagal tone which was related to respiration (Julian, 1983; Abdel-Rahman et al., 2010). Moreover, the cardioxic effects noticed in this communication indicate that *A. bicolor* venom has a sympathomimetic effect by releasing a massive amount of catecholamines from the sympathetic nerve endings in the heart. The outpouring of catecholamines in response to the scorpion sting is probably a major factor in the pathogenesis of ST-T changes (Bawaskar and Bawaskar, 1992). Catecholamines increase myocardium oxygen consumption, aggravate the ischemic state and create electric instability (Krishna Murthy, 2000). Cellular hypoxia (usually due to ischemia) slowed SA and AV nodal conduction leading to bradycardia (Senges et al., 1979). The occurrence of premature ventricular contraction (ventricular ectopic beats) was also observed. This is probably due to the venom induced dissociation in the heart conduction system and to the catecholamine discharge after scorpion envenomation (Ismail, 1995; Krishna Murthy, 2000). Infarction represented in ST segment elevation also appeared after the application of *A. bicolor* venom. This disorder could be attributed to a leakage of K⁺ from the treated myocardium into the interstitial fluid due to inhibition of sarcolemmal Na,K-ATPase (Krishna Murthy, 1982; Ismail, 1995). In conclusion, we describe the first *in vitro* investigation about cardiotoxic effects of *A. bicolor* venom using the heart of toads. Our electrophysiological data revealed that the direct effect of *A. bicolor* venom on heart muscles constitute a crucial role in the development of heart toxicity and disorders. Moreover, venomics studies are needed to characterize venom peptides.
responsible for the induction of negative chronotropism and positive inotropism which could be good templates for developing novel therapeutic agents for heart diseases.

Competing interests

The authors declare that there are no conflicts of interest.

References


