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Short communication

Distinct cardiotoxic effects by venoms of a spitting cobra (*Naja pallida*) and a rattlesnake (*Crotalus atrox*) revealed using an *ex vivo* Langendorff heart model

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ABSTRACT

Here we describe the acute myocardial effects of an elapid (red spitting cobra, *Naja pallida*) and a viper (western diamondback rattlesnake, *Crotalus atrox*) venom using an *ex vivo* heart model. Our results reveal two different pathophysiological trajectories that influence heart function and morphology. While cobra venom causes a drop in contractile force, rattlesnake venom causes enhanced contractility and frequency that coincides with differences in myocellular morphology. This highlights the medical complexity of snake venom-induced cardiotoxicity.

1. Introduction

Snakebite envenoming is an important but neglected tropical disease with estimated annual mortality rates around 81,000–138,000 (Gutiérrez et al., 2017). The medically relevant snake species can generally be classified into two distinct families: *Elapidae* and *Viperidae*. Elapid venoms are dominated by enzymatic group I phospholipases (PLA_2s) and non-enzymatic three-finger toxins (3FTxs), viper venoms are typically dominated by enzymatic toxins, such as group II phospholipases (PLA_2s), snake venom serine proteinases (SVSPs) and snake venom metalloproteinases (SVMPs) (Tasoulis and Isbister, 2017). While these snake families are known to cause pathologies differentially, there is little known on their specific cardiotoxic effects (Gutiérrez et al., 2017; Averin and Utkin, 2021; Fry, 2015).

Venom-induced cardiotoxicity is a regular complication after snakebite envenoming, which may have serious cardiovascular consequences including hypertension, hypotension, brady- or tachycardia, atrial fibrillation, cardiac arrest, and myocardial infarction (Kakumanu et al., 2019). These pathophysiological effects are caused by either reversibly or irreversibly disrupting key functions related to the heart and/or cardiovascular system. Various toxin classes have been known for causing cardiovascular effects (Averin and Utkin, 2021). Kakumanu et al., performed comparative *in vivo* rat experiments using a single elapid species (i.e. *Pseudonaja textilis*) and six viper species (*Bitis* spp., *Crotalus* sp., *Daboia* spp. and *Echis* sp.) revealing two distinct trajectories of cardiovascular effects: prolonged hypotension or rapid cardiovascular collapse (Kakumanu et al., 2019). Several studies focused on cardiotoxic effects of snake venom, however a direct comparison between elapids and vipers using an *ex vivo* heart function assay has not been performed to date. The *ex vivo* Langendorff apparatus enables to study isolated cardiac function, creating a controlled setting for precise evaluation of snake venom-induced cardiac effects excluding complex physiological interference of whole body systems. This allows immediate observation of acute impacts on the contraction-relaxation cycle and force development. Notably, this study is the first to demonstrate *N. pallida* and *C. atrox*'s functional influence on cardiac dynamics. These distinctive

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assessments cannot be run *in vitro* or *in vivo* systems and opens therefore avenues for further research, providing insights into functional consequences of snake venom on the heart. We assessed the cardiovascular effects of a representative elapid species (i.e., red spitting cobra, *Naja pallida*) and a representative viper species (i.e., Western diamondback rattlesnake, *Crotalus atrox*) using a combined *ex vivo* Langendorff and histological approach. *Naja pallida* venom is dominated by group I PLA_2s and 3FTxs (Kazandjian et al., 2021) and cobra venom and their isolated components (e.g., 3FTx cardiotoxins) have been shown to cause *in vitro* and *ex vivo* cardiotoxic effects (Averin et al., 2022; Cher et al., 2005; Nayler et al., 1976; Sarkar et al., 1942; Sarkar, 1947). *Crotalus atrox* venom is dominated by group II PLA_2s, SVSPs and SVMPs (Calvete et al., 2009), and rattlesnake venoms and their isolated components (e.g., atrotoxin) are known to cause *in vitro* and *in vivo* cardiotoxicity (Kakumanu et al., 2019; Gilliam et al., 2012; Slade et al., 2021; Hamilton et al., 1985; de Paola and Rossi, 1993; Sartim et al., 2023). Here, we reveal differential myocardial function and morphology caused by an elapid and viper venom using an *ex vivo* heart function model, suggesting that distinct venomous snake families cause different cardiovascular effects via distinct molecular mechanisms.

2. Materials and methods

2.1. Animals

All animal experiments were performed with approval of the Animal Care and Use Committee of the Utrecht University, The Netherlands. All experiments were conducted according to the directive 2010/63/EU for animal experiments. Surplus adult male Wistar rats (300–400 g) were used in surgical training sessions for biotechnicians under this permit. After these standardized training sessions, the rat hearts were removed and used in the Langendorff set up.

2.2. Chemicals

The venoms of red spitting cobra (*Naja pallida*) and western diamondback rattlesnake (*Crotalus atrox*) and were sourced from the extensive library of the Faculty of Science, BioAnalytical Chemistry, Vrije Universiteit Amsterdam (VU). All venoms were sourced prior to October 2014; therefore, these do not fall under the Nagoya Protocol (Secretariat, 2011). Venoms were lyophilized directly after milking and stored at −80 °C. Samples were reconstituted in milliQ (mQ) H_2O to the desired stock solutions, were then aliquoted and subsequently snap frozen in liquid nitrogen and stored at −80 °C until use. During the experiment, the Eppendorf reaction vessels containing venom were kept on ice and allowed to thaw shortly before use at room temperature (20 °C). Then, 100 μg venom was dissolved in 300 μl Krebs-Henseleit buffer (KHB). KHB was prepared on the day of the experiment. The following components were dissolved in 1L of demineralized water: NaCl 75 g, KCl 3.5 g, CaCl2 1.5 g, NaH_2PO_4 0.5 g, $MgCl_2$ 1 g, glucose 2 g and $NaHCO_2$ 1.7 g. This buffer was used to supply the heart with nutrition whereas carbogen (95 % O_2 and 5 % CO_2) keeps the acidity stable. The perfusion fluid was heated to 38.0 °C, which was consistent with the rat heart temperature.

2.3. Heart preparation

Rats were anesthetized with medetomidine (50 μg/100 g body weight) and ketamine (7.5 mg/100 g body weight) via an intra peritoneal injection. After the surgical training session, the anesthetized rats were humanely euthanized by decapitation. Subsequently, the heart preparation was performed as soon as possible (±2.5 min per preparation) to prevent loss of function due to oxygen deprivation and to prevent formations of blood clots in the coronary vessels. To avoid this coagulation, heparin (125 IU/0.5 ml) was administered through the jugular vein catheter shortly before euthanization of the animal. A hole was cut in the aorta of about 1 cm from the origin to be able to insert the cannula filled with heparin (125 IU/ml) at 3–5 mm just before the aortic valves. This makes the perfusion fluid flow backward through the heart using the coronary vessels that emerge in front of these valves (retrograde perfusion). Once the cannula was placed correctly, the color of the heart changed from deep red to pale. The heart was then placed in the Langendorff setup. Hearts were allowed to equilibrate for maximally 15 min.

2.4. Langendorff setup

The Langendorff setup allowed to measure contractile force and heart rate (frequency) as the stimulus to rhythmically contract originated in the heart itself via the pacemaker system (Liao et al., 2012). The beating heart was connected to the displacement transducer with an apex hook. Vertical displacement of the apex was a parameter for frequency and contractile force. In this study, contractile force is the primary parameter, as it is an important characteristic of muscle function. Another physiological parameter that was measured was cardiac frequency.

2.5. Determination and analysis of contractile force and frequency

The last 5 min of the 15 min acclimatization period were considered to be the baseline measurement was consistent between experimental groups. The rat hearts were divided into three groups, a group treated with *Naja pallida* venom (n = 5), a group treated with *Crotalus atrox* venom (n = 5) and a control (KHB-buffer-treated) group (n = 5). From here on referred to as control group. After baseline measurement, a 100 μg dose of venom was administered to the hearts via the canula. Subsequently, the canula was rinsed immediately with 300 μl KHB alone. For the control group only 300 μl KHB was administered to the heart via the canula. Because of retrograde perfusion across the coronary system, the exposure of the myocardium to the venom or buffer lasted one contractile heart cycle. The effect of this short-term exposure was monitored for 5 min. Contractile force was calculated for each minute during this 5 min window by assessing the difference in peak height compared to baseline measurement. Contractile frequency was assessed by taking the number of peaks per minute compared to baseline.

2.6. Histological analysis

After the functional physiological analyses, the rat heart was cut at the transversal plane containing the right ventricle, septum and left ventricle. The tissue was fixated in 4 % paraformaldehyde. From the embedded tissue, 4 μm thick sections were cut which were subsequently stained with Hematoxylin-Eosin (H&E) for light microscopic assessment according to standard procedures. Digital markings were applied to the specimens to indicate specific locations in the heart, such as the left and right ventricle and septum. For each location, 15 photographs were taken at a final magnification of 400×. Only transverse cardiomyocytes with a visible cell nucleus were used in the analyses. For each location of the heart, nine areas were assessed for snake venom-induced effects.

2.7. Data analysis

All results are indicated as mean ± standard deviation (SD) and statistically analyzed. Differences between groups were determined with a Kruskal-Wallis non-parametric test or a paired T-test. A p-value of <0.05 is considered statistically significant. GraphPad Prism version 9 was used in these analyses.

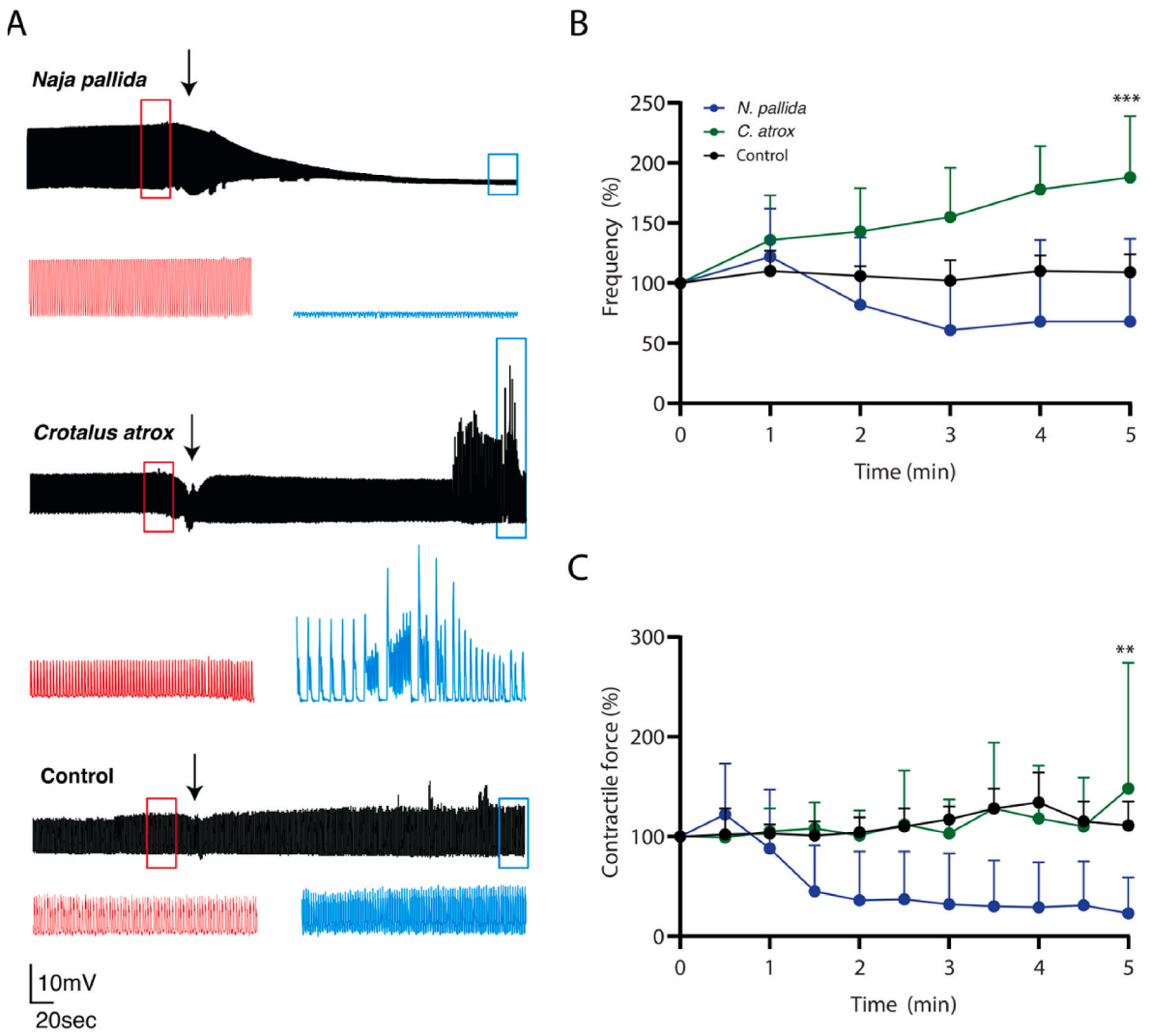

Fig. 1. Typical Langendorff tracings after administration of (A) *N. pallida* venom (100 μg/300 μl); *C. atrox* venom (100 μg/300 μl); Control (300 μl). Arrows indicate the time of administration of the venom or KHB-buffer. The tracings are representative (n = 5) for each group. Using these tracing, the contractile force (1B) and frequency (1C) of an isolated rat heart for 5 min were determined. Data are presented as mean ± SD normalized to control and significant differences between groups were determined with a Kruskal-Wallis non-parametric test or a paired T-test (**p < 0.05; also indicate here between which observations it is significant).

3. Results

3.1. Effect of true cobra and rattlesnake venom on functional heart physiology

We investigated the cardiotoxic effects of two clinically-relevant snake venoms on cardiac function. *Ex vivo* rat hearts were exposed to red spitting cobra (*Naja pallida*) and western diamondback rattlesnake (*Crotalus atrox*) venom (100 μg/300 μl; n = 5 per species) compared to KHB buffer (300 μl; n = 5) as control group. Fig. 1A shows representative tracings of the two venoms compared to a normal heart (control) and allows to study both the contractile force (Fig. 1B) and frequency (Fig. 1C). During the 5-min experimental period, *Naja pallida* venom abrogated the heart contractile force (32 % ± 36 %; p < 0.05 compared to control at 5 min), whereas the hearts remained unaffected when exposed to *Crotalus atrox* venom (Fig. 1B). In contrast, *Naja pallida* did not affect contraction frequency (68 % ± 69 %), but two *N. pallida*-treated hearts completely stopped contracting, resulting in the relatively large standard deviation in data. Hearts that were exposed to *Crotalus atrox* venom showed increased frequency (188 % ± 51 %; p < 0.05 vs. control at 5 min; Fig. 1C). The control hearts that we only exposed to KHB had no effect on either force (96 % ± 24 %; Fig. 1B) or frequency (87 % ± 50 %; Fig. 1C). This suggests that the tested elapid and viper venoms show distinct trajectories in causing cardiotoxicity.

3.2. Histological analyses that are linked to functional observations

To determine whether the different effects on cardiac function are underpinned by different changes in cellular morphology, we performed histological analyses. Transversal slides of the left ventricular wall, septal wall and the right ventricular wall were stained with H&E to assess morphological changes in the myocardial tissue. Compared to the control tissue, hearts treated with *N. pallida* venom showed areas that were both unaffected and affected. In the latter case diffuse moderate to severe interstitial and perivascular edema was observed. In addition, severe multifocal to diffuse hyaline and homogeneous cytoplasm with loss of cross striations (hyaline degeneration), fragmentation (Zenker's necrosis) and vacuolation (vacuolar degeneration) was observed intra- and intercellularly (Fig. 2 A, B, C). These observations were made in the left and right ventricle, as well as in the septal region and likely found its origin in the retrograde perfusion of venom through the three branches of the coronary artery system across these areas of the heart (Kainuma et al., 2017). The histopathology lesions of the heart cells likely supports the observed reduced cardiac pump function in the heart function assay

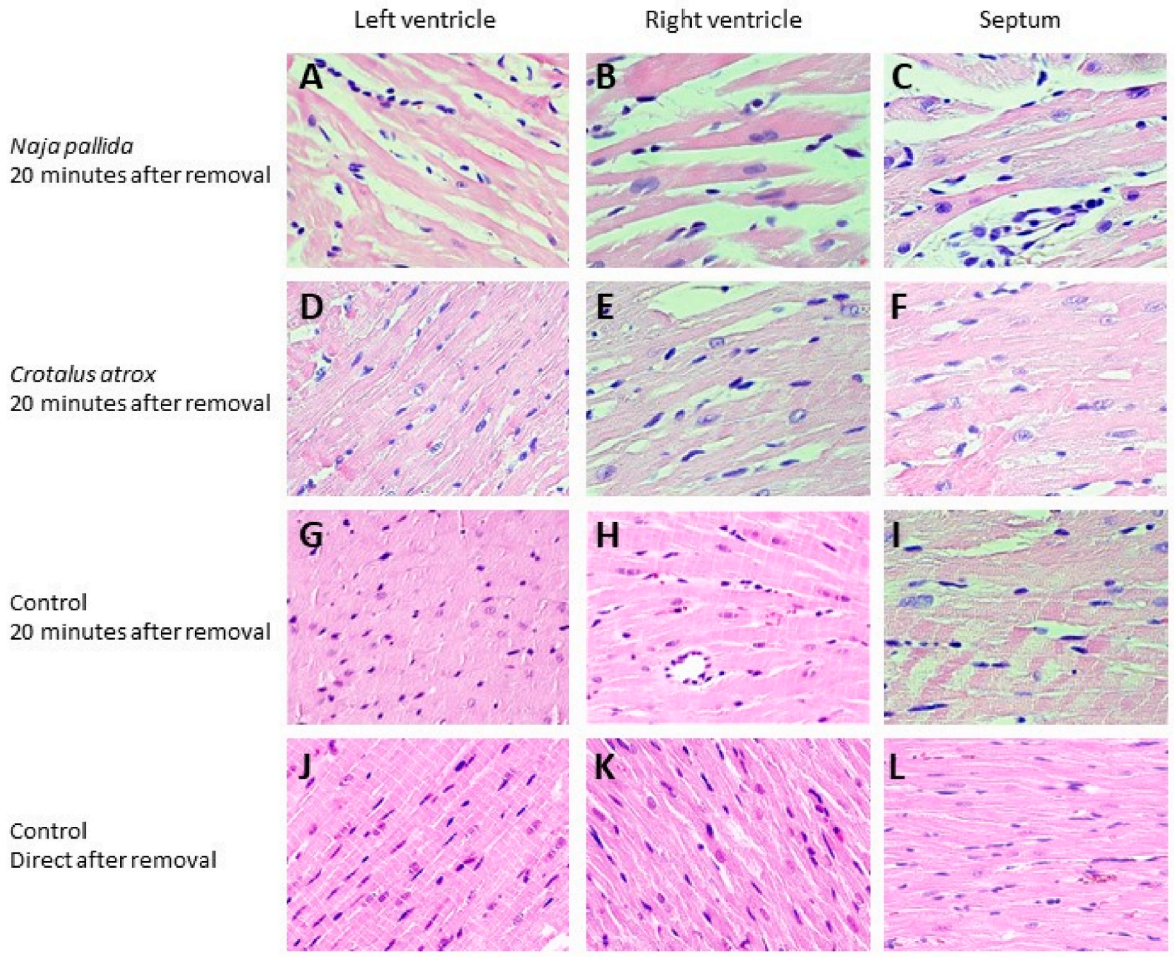

Fig. 2. Microscopic examination of myocardial left ventricular (LV; A, D, G, J), right ventricular (RV; B, E, H, K) and septal (S; C, F, I, L) sections of H&E-stained tissues. The *N. pallida* (A, B, C) treated hearts showed focal patches of cells with also called 'tombstone' appearances. The cytoplasm in these hearts was more condensed and the nuclei shrunk (pyknosis) or even disappeared (karyorrhexis). Granulation tissues were lacking between the cells. The *C. atrox* treated hearts (D, E, F) showed a less deteriorated phenotype compared with *N. pallida* treated hearts (A, B, C). Nevertheless, there are signs of deteriorated in cell-cell interaction relative to control hearts (at 20 min after removal G, H, I) and certainly relative to directly extracted hearts (J, K, L). The venom-induced observations in the hearts were not site-specific, but were observed in the LV, RV as well as in the septal region.

(Lempek et al., 2022). The very short exposure time of the venom to the myocardial tissue (as the venom only passes once through the heart), makes it very likely that the observed effects are transmitted to cardiomyocytes via the coronary system and stresses the potent activity of the venom. In the *Crotalus atrox*-exposed hearts moderate interstitial perivascular edema was observed as well as moderate vacuolation and fragmentation (Fig. 2 D, E, F). These observations do not directly support the observed increase in frequency, but do give rise to the hypothesis that both venoms affect cardiac function via a different mechanism.

An advantage of the current *ex vivo* model is that other hematological factors present in the vascular system do not interfere in the observed effects. The observed effects are therefore a direct consequence of exposure to the *Naja pallida* venom via the coronary system. As previously suggested by an *in vitro* cell culture experiment of Hamilton et al., 1985) (Hamilton et al., 1985), voltage-dependent calcium influx in cardiac muscle cells may contribute to the observed increase in strength and frequency in *Crotalus atrox*-exposed hearts. Next, analyses on histomorphological levels show signs of deteriorated cell-cell interaction in *Crotalus atrox*-exposed hearts, but to a much lesser extent than that visible in the *Naja pallida*-treated hearts. The exact mechanism how *Crotalus atrox*-venom affects acute cardiac function will require further investigations.

In summary, while this study presents valuable insights into the distinct cardiotoxic effects of *Naja pallida* and *Crotalus atrox* venoms, addressing the outlined limitations and exploring the suggested future directions could enhance the comprehensiveness and applicability of these findings in the broader context of snakebite envenoming. Further exploration into the molecular mechanisms underlying venom-induced cardiac effects will be necessary. Investigating specific venom components responsible for alterations in contractile force and frequency, as well as their interactions with cardiac cellular pathways, would advance the understanding of the pathophysiology involved. However, extrapolating findings to the *in vivo* context should be done cautiously, considering the absence of systemic factors present in a living organism. Future studies will focus to broaden the scope by including a diverse range of snake species from both elapid and viper families. This would facilitate a more nuanced comparison of venom-induced cardiac effects across different genera and species, contributing to a comprehensive understanding of snakebite envenoming and to further elucidate the complexity of (cardio)toxicity caused by snake bites with potential consequences for medical treatment.

Ethical statement

All animal experiments were performed with approval of the Animal

Care and Use Committee of the Utrecht University, The Netherlands. All experiments were conducted according to the directive 2010/63/EU for animal experiments.

CRediT authorship contribution statement

Ronald Vlasblom: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jory van Thiel:** Writing – review & editing, Visualization, Validation. **Matyas A. Bittenbinder:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Jon-Ruben van Rhijn:** Visualization, Methodology, Formal analysis, Data curation. **Rinske Drost:** Writing – review & editing, Methodology. **Lotte Muis:** Methodology, Investigation, Formal analysis. **Julien Slagboom:** Methodology, Investigation. **Daniela Salvatori:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Jeroen Kool:** Writing – review & editing, Supervision, Conceptualization. **Robert Jan Veldman:** Writing – review & editing, Visualization, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The silhouettes of the snakes in the graphical abstract were created by Gabriela Palomo-Munoz (*Crotalus*) and by V Deepak (*Naja*)

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