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An *in vivo* assessment of inflammatory and oxidative stress responses in *Echis ocellatus*-venom induced cardiotoxicity

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ABSTRACT

Echis ocellatus is one of the many viper species that accounts for severe pathophysiological alterations in tissues of organs after envenoming. However, limited information regarding the potential cardiac toxicity due to viper envenoming is available. This current study investigated cardiotoxicity associated with *E. ocellatus* envenoming in rat model. Twenty (20) male Wistar rats weighing between 140 and 180 g were divided randomly into two groups (n = 10). Rats in group 1 (control) were injected with saline while rats in group 2 were envenomed intraperitoneally with 0.055 mg/kg^{-1} ($LD_{6.25}$) of *E. ocellatus* venom. The rats were envenomed on day 1 with a repeated dose administered on day 15, afterwards the animals were monitored till day 30. The venom caused significant ($P < 0.05$) reduction in body and heart weights including the heart index of envenomed rats compared to the control. Levels of malondialdehyde significantly ($P < 0.05$) increased with decrease in glutathione concentration and catalase activity in heart tissues of envenomed rats. *E. ocellatus* venom elevated pro-inflammatory cytokines response as levels of tumor necrosis factor-alpha and interleukin1-beta significantly ($P < 0.05$) increased in cardiac tissues of the envenomed rats compared to control. The venom induced severe morphological defects in the heart tissues of envenomed rats indicating that *E. ocellatus* venom could actuate cardiotoxicity post envenoming.

Introduction

In recent times, cardiac failure manifesting from dysregulation of cardiac function has been a leading cause of heart failure which most times result in death and disability [1]. Snake venom toxins are known to target the cardiovascular system with detrimental effects on tissues of organs after envenoming [2]. Several studies have reported systemic toxicity resulting from haemotoxic, cytotoxic and neurotoxic activities exhibited by snake venom toxins after envenomation [3]. However, cardiotoxic activities associated with snakebite envenoming have not attracted significant research attention. Manifestation of cardiac toxicity is mostly associated with snakebite envenomings from the Viperidae and Elapidae families. Cardiac toxicities caused by vipers and elapids envenomings can be attributed to snake venom toxins such as phospholipase A2 and cardiotoxins which are well known for their direct cardiovascular effects after envenomation [4,3].

Vipers constitute species of most medically important snakes worldwide [5] and their envenomings are accompanied with severe pathophysiology which are usually fatal [6]. *Echis ocellatus* also, called Carpet Viper belongs to the family Viperidae, a specie responsible for more fatalities when compared to any other vipers in Africa annually [6]. Envenoming by *E. ocellatus* is often characterized by severe local effects such as inflammation, necrosis, haemorrhage, oedema, and pain. These local effects often result to permanent disabilities while the systemic effects associated with *E. ocellatus* envenoming are accompanied with spontaneous bleeding, hypotension, hemostasis disorder and shock [7].

E. ocellatus venom contains numerous toxic proteins with metalloproteinase and phospholipase A2 as major enzymes that targets various systems of the body [8]. Metalloproteinase enzymes are broadly categorized as cytotoxic and haemotoxic as they typically target the circulatory system causing hemostasis disruptions such as coagulation, fibrinolysis, thrombosis and variable tissue damage [8]. On the other hand, phospholipase A2 is myotoxic in nature and targets skeletal muscles, neuromuscular junctions, haemostatic and cardiovascular systems [9,10].

E. ocellatus have been implicated in cardiotoxicity which is usually observed in patients dying of severe viper bites [11]. Complications

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associated with cardiac dysfunctions following viper envenoming may occur in forms of cardiac arrhythmia, myocardial infarction and changes in the electrocardiogram [11–13]. Other notable cardiovascular effects associated with snakebite envenoming include hypotension, cardiac arrest, hypertension and atrial [14,15].

Snake venom toxins have been linked directly as initiator of cardiac dysfunction by inducing oxidative stress (OS) which is triggered by excessive production of reactive oxygen species (ROS) [16]. Studies have reported that increased OS resulting from oxidant damage have deleterious effects on different organs structures and functions including the cardiac organ [17,18]. Also, ROS had been established to be directly involved in inflammatory reactions with damaging effects on cellular physiology and plays a significant role in various pathological conditions [19]. Furthermore, studies have documented that inflammation triggered by elevated inflammatory cytokines strongly predict the manifestations of heart diseases such as coronary artery disease, myocardial infarction and heart failure [20]. Despite these established reports, limited scientific information is available regarding cardiotoxic effects arising from *E. ocellatus* envenoming. Therefore, this study is aimed at providing more scientific insight on the potential cardiac toxicity resulting from *E. ocellatus* bite post envenoming in rat model.

Materials and methods

Chemicals and kits

Rats Enzyme-Linked Immunosorbent Assay (ELISA) Kits for TNF-a and IL-1β assays were manufactured by PeproTech, Inc. London, UK. Other reagents and chemicals were of good grade and purchased from Sigma-Aldrich, Inc, USA.

Venom collection

A lyophilized venom pooled from an adult *E. ocellatus* snake was procured from herpetarium of the Department of Veterinary Physiology and Toxicology, Amadu Bello University, Zaria, Nigeria. The venom sample was transported to the laboratory of the Department of Zoology, Osun State University, Osogbo, Nigeria at maintained temperature of 4 °C and stored until required.

Experimental animals

Animals used for this study were twenty male albino Wistar rats weighing between 140 and 180 g. The rats were obtained from the animal facility of the Department of Zoology, University of Ibadan, Nigeria and transported to the laboratory of the Department of Zoology, Osun State University, Osogbo, Nigeria using a well-ventilated transparent plastic cages. The animals were acclimatized at room temperature (25 ± 2 °C) in the laboratory for two weeks and fed with standard rat pelletized feed with water provided *ad libitum*. The experimental protocols for the study was approved by the University of Ibadan-Animal Care and Research Ethics Committee (UI-ACUREC) with assigned number: UI-ACUREC: 18/0108. The animal experiment complied with the National Research Council's publication on guide for the care and use of laboratory animals [21].

Study design and envenoming procedures

Animals were divided randomly into two experimental groups of ten rats each. Rats in group 1 were injected with 0.2 ml of saline (Control). The lethal dose (LD_{50}) of *E. ocellatus* venom as determined in our previous study was 0.22 mg/kg^{-1} [22]. However, rats in group 2 were injected intraperitoneally with 0.2 ml of 0.055 mg/kg^{-1} ($LD_{6.25}$) of *E. ocellatus* venom to minimize casualties in this group due to high toxicity of the venom. The rats were envenomed on the first day and repeated on the fifteenth day while the experiment lasted for thirty days. Mortality and clinical signs of toxicity were observed and recorded accordingly.

Body weight

Experimental rats were weighed pre-envenomation (initial weight) and before being sacrificed on the last day (terminal weight). The body weight gain was calculated using the formula:

$$Body\ weight\ gain = \frac{Terminal\ weight\ of\ rats - Initial\ weight\ of\ rats}{Initial\ weight\ of\ rats} x100$$

Organ collection

The experimental rats were thereafter sacrificed through cervical dislocation following guides [23]. The heart of each rat was surgically removed and measured to determine the organ weight and relative organ weight. Thereafter, biochemical analyses and histological examinations were carried out on the organs. The relative heart weight was determined using the formula:

Relative organ weight = 2.4 Oxidative stress determination in the heart

Measurement of lipid peroxidation

Induction of oxidative damage in the heart was determined by measuring the extent of lipid peroxidation (LPO) in the tissue sample using estimated thiobarbituric acid reactive substances (TBARS) [24]. An aliquot of the sample (1.0 mg protein) was added to tubes containing 1.5 ml of acetic acid (pH 3.5, 20 % v/v), SDS (8 % w/v, 0.2 ml) and 1.5 ml thiobarbituric acid (0.8 % w/v). The mixture was then heated in a boiling water bath for 45 min. The adducts formed were extracted into 1-butanol (3 ml) and the absorbance of TBARS formed were taken at 532 nm.

Measurement of reduced glutathione content

Levels of reduced glutathione in the heart was measured as described [25]. GSH reacts with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) or Ellman's reagent to generate 2-nitro-5-thiobenzoic acid and glutathione disulphide (GSSG), a yellow-coloured compound measured spectrophotometrically at 412 nm.

Measurement of catalase activity

Catalase activity in the heart was determined by measuring the rate of hydrolysis of H_2O_2 at 240 nm [26]. Hydrogen peroxide (H_2O_2) 8.8 mM in sodium phosphate buffer (0.1 M, pH 7.0) was added to 0.05 mg protein of tissue samples. The decrease in absorbance was monitored for 3 min and the activity was expressed as l mol H_2O_2 decomposed/min/mg protein (ϵ-43.6/mM/cm).

Estimation of pro-inflammatory cytokines release

Preparation of tissues samples

Sample of frozen heart tissues were homogenized in a 1.5 ml RIPA buffer (25 mM TrisHCl, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS pH = 7.6) supplemented with Protease inhibitors at 4 °C. The homogenate was incubated on ice for 30 min and then centrifuged at 10,000 × g for 30 min at 4 °C. Following centrifugation, the supernatants were transferred to labelled Eppendorf and stored at −80 °C for cytokine measurement.

Measurement of tumor necrosis factor-alpha (TNF-α) and interleukin1-beta (IL-1β) production

Quantitative measurement of the level of cytokines was performed using mini enzyme linked immunosorbent assay (ELISA) development

kits (Peprotech). Well plates were set up according to the manufacturer's instructions and read using an ELISA plate reader at 405 nm with 650 nm as the correction wavelength. Concentrations (pg/mL) of TNF-α, and IL-1β in heart tissues were estimated.

Histopathological evaluation

Histopathological examination of the heart tissues was carried out using standard laboratory procedures. The heart tissues of the rats were fixed in Bouin's solution, embedded in paraffin, sectioned into 4 μm thicknesses and placed on microscopic slides. Slides were observed using the light microscope at a magnification of x400 after staining with haematoxylin and eosin [27].

Statistical analysis

Statistical analysis Values were expressed as mean ± standard error of mean (SEM) and analyzed using T-Test to compare the significant ($P<0.05$) differences between the control and test group. An independent sample test was used for comparison. Data were analyzed using GraphPad Prism software, version 7.0 produced by GraphPad Software, San Diego, CA, USA.

Results

Clinical signs of toxicity and mortality

Local haemorrhage was noticed at the site of venom injection in rats of the envenomed group combined with sluggishness and low appetite post envenomation while the control rats were active and fed well. Death was not recorded in the control group however, mortality was recorded on day 15 and day 19 of the experiment in envenomed group (Table 1).

Body weight gain, heart weight and heart index of envenomed rats

Values obtained for the body weight gain of the control was significantly ($P < 0.05$) higher compared to the envenomed group. Also, the venom caused a significantly ($P < 0.05$) reduction in heart weight and index of envenomed rats compared to the control (Fig. 1).

Oxidative stress parameters

MDA levels in heart tissues of envenomed rats was significantly ($P < 0.05$) higher compared to the control. GSH content in heart of envenomed rats significantly ($P < 0.05$) decreased when compared to the control. The same trend was observed in catalase activity of the heart as there was significant ($P < 0.05$) decline in enzyme activity of envenomed rats compared to the control (Table 2).

Pro-inflammatory cytokines production

The venom enhanced pro-inflammatory cytokines release with significant ($P < 0.05$) increase in interleukin1-beta (IL-1β) response in the heart tissues of envenomed rats compared to the control. Production of tumor necrosis factor-alpha (TNF-α) in heart tissues of envenomed rats showed a significant ($P < 0.05$) elevation in relation to the control (Fig. 2).

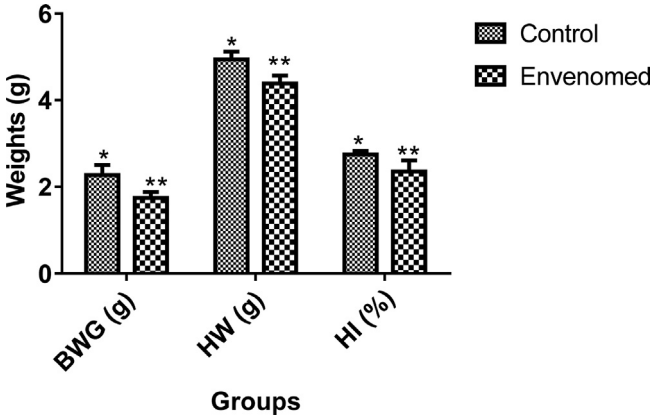

Fig. 1. Body weight gain (BWG), heart weight (HW) and heart index (HI) of experimental rats. Data are represented as mean ± SEM (n = 5). Values in the same column with different superscript are considered significant ($P < 0.05$).

Table 2
Effects of *E. ocellatus* venom on antioxidant enzymes of experimental rats.

Groups	CAT (kU/mL)	GSH (nmol/g tissue)	MDA (nmol/g tissue)
Control	2701.00 ± 90.48^{b}	2.96 ± 0.41^{b}	3.39 ± 0.26^{a}
Envenomed	1830.80 ± 221.35^{a}	2.03 ± 0.10^{a}	8.50 ± 0.47^{b}

Data are expressed as mean ± SEM, (n = 5). Values in the same column with different superscript are considered significant ($p < 0.05$).
CAT: Catalase, **GSH:** Glutathione, **MDA:** Malondialdehyde.

Histopathological examination of the heart

Examination of histological slide prepared from the heart tissue of the control revealed normal appearance of cardiomyocytes with no structural defects whereas slide of heart tissue from the envenomed group showed attrition of cardiomyocyte fibers, presence of mild epicardia hemorrhagic foci, a patchy loss of myofiber striations, inflammatory cells and aggregates of satellite cell accumulation (Fig. 3).

Discussion

Snake venom toxins have potential damaging effects on system functions of the body particularly the cardiovascular system due to the presence of complex protein mixtures and enzymatic components, that exhibit diverse and multifactoral pathological characteristics [2,11]. These snake venom proteins possess numerous pharmacological actions including direct effects on organs such as the heart [9,28]. Also, viper venoms are known to cause cardiac toxicity after envenomation due to the presence of metalloproteinase enzyme that target the circulatory system including the heart which is a major organ of blood circulation [4,8].

Table 1
Mortality occurrence in the envenomed group during the thirty days experimental period.

Groups	Envenomation	Day 1	Day 15	Day 19	Day 30	Mortality (%)
Control	–	–	–	–	–	0.00
Envenomed	–	–	1	1	–	20.00

Number of rats per group (n = 10).

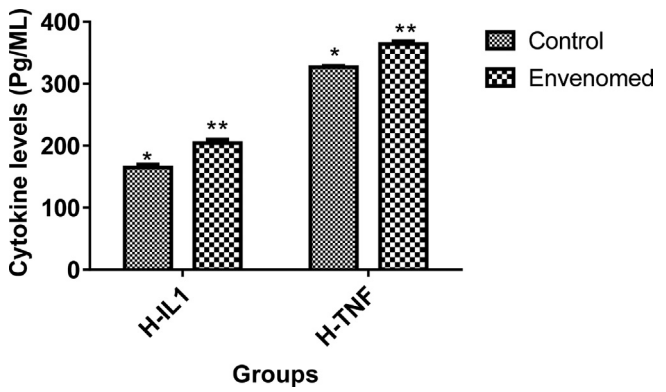

Fig. 2. Interleukin1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) production in heart tissues of envenomed rats H-IL1: Heart Interleukin1-Beta, H-TNF: Heart Tumor necrosis factor-alpha. Data are expressed as mean ± SEM, (n = 5). Values with the same superscript are considered not significant ($p < 0.05$).

In this study, the animals were injected with a low dose ($LD_{6.25}$) of *E. ocellatus* venom through the peritoneal route to minimize fatalities after envenomation. Also, the rats were envenomed twice at intervals to initiate the pathophysiological effects expected of a viper venom as earlier done in our previous study [29]. The venom caused toxicological effects on the cardiac tissues of the envenomed rats such as significant decrease in heart weight and index. These effects were evidences of the direct interactions of the venom toxins with the heart tissues that result in cardiac toxicity in envenomed victims [9]. It is pertinent to note that the sub-lethal dose of the venom used in this study had been reported to cause direct toxicity to vital organs in envenomed rats in our previous study [29].

It has been well established that toxins present in viper venom are more typically associated with induction of oxidative stress (OS) through elevation of oxidative stress indicators such as lipid peroxidation [16,30]. Dysregulation between the production of reactive oxygen species (ROS) and the endogenous antioxidant defense mechanisms could result to OS. Also, excessive generation of ROS are linked to multiple pathophysiological pathways in the heart [18]. In this study, the venom enhanced lipid peroxidation (LPO) in the heart of envenomed rats indicating OS status. Excessive generation of ROS may be attributed to the observed increase in LPO level, which is a consequence of oxidant damage. This observation was in tandem with other studies with report that manifestation of OS could be as a result of upsurge in ROS production or breakdown of the cellular antioxidant system [17,31].

Glutathione (GSH) is regarded as the main natural antioxidant defense system present in most living cells which acts to scavenge or neutralize ROS effects either in a direct way or through enzymatic catalysis [32]. However, GSH deficiency could result in damage of the antioxidant defense system leading to significant elevation of lipid peroxidation [31] as observed in this study. Our findings aligned with previous interpretation of consequences of GSH deficiency in a biological system as LPO levels increased with diminished effect on GSH content in the heart of the envenomed rats. Studies have revealed that exposure of the heart to free radicals resulted in a decline in high energy phosphates, loss of contractile function and caused structural abnormalities [33].

Furthermore, toxic enzymes of viper venom have been implicated in multiple pathologies that contributed to oxidative stress [20]. However, the mechanism underlying the significant increase in LPO resulting in OS is not yet clear. Although, studies have postulated mechanisms that could be liable for OS induction after viper envenoming. Katkar et al. [34] has reported that increase in polyunsaturated fatty acids could be a possible reason for elevation of LPO following administration of viper (*E. carinatus*) venom. Other mechanistic possibility is the depressive attribute of free radicals on mitochondrial respiration, cytohrone oxidase and glucose-6-phosphate which facilitated increase in the levels of malondialdehyde, an indicator of free radical-induced lipid peroxidation [33]. There could be a similar possibility of peroxidation manifesting as a result of *E. ocellatus* envenomation in this study.

Cytokines are inflammatory mediators that coordinate inflammatory responses and contribute a significant role in inflammation [20]. Cytokines such as interleukin IL-1, IL-6, IL-8, TNF-α, IL-16, and IL-17 are known to trigger acute inflammation [35]. In this study, *E. ocellatus* venom caused a significant up-regulation of TNF-α and IL-1β in heart of envenomed rats when compared to the control, which is an indication of venom induced inflammation on the cardiac tissues. This was in tandem with our previous findings on inflammatory responses initiated on cardiac tissues of rats envenomed with sub-lethal dose (LD_{50}) of *E. ocellatus* venom [22]. Consequently, inflammatory effects could facilitate the development of heart failure or directly harm the heart resulting in diverse cardiovascular diseases [36]. According to Tian et al. [37], increase in the level of pro-inflammatory cytokines such as TNF-α and IL-1β have been linked to several heart diseases including coronary heart disease, atherosclerotic heart disease and congestive heart failure.

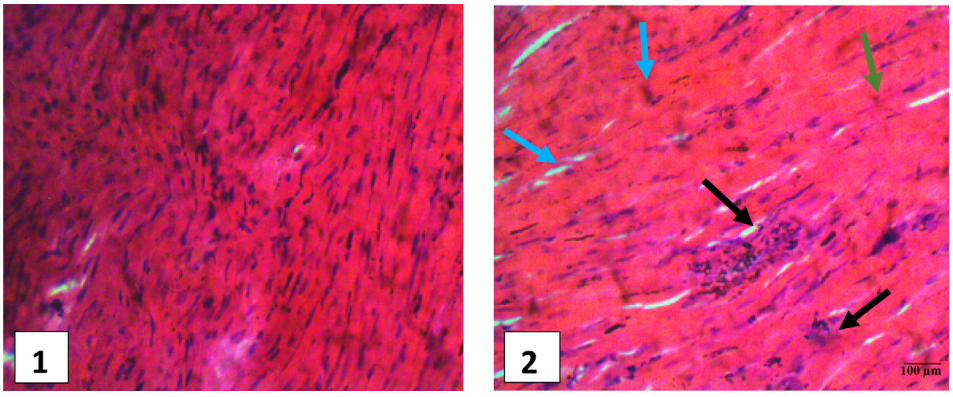

Fig. 3. Histological examination of the heart. Control (1): A normal appearance of the cardiomyocytes, vascularized with no observable defect. Envenomed group (2): The slide revealed attrition of cardiomyocyte fibers, presence of mild epicardia hemorrhagic foci (blue arrows), a patchy loss of myofiber striations (green arrow), inflammatory and satellite cell accumulation (black arrows). Hematoxylin and Eosin staining (x400). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, TNF-α is produced in the myocardium under volume overload and higher levels has been found present in failing myocardium in patients with chronic heart failure [38,39]. Likewise, IL-1β is an important inflammatory mediator that is released at the sites of injury and recruits cellular partners**.** TNF-α initiates the synthesis of inactive-IL-1β which is subsequently cleaved by caspase 1, a cysteine protease activated by inflammasomes [40] that is overtly expressed in patients with heart failure [41]. The up-regulation of IL-1β and TNF-α production in the heart tissues of envenomed rats was indicative of *E. ocellatus* venom ability to initiate cardiac inflammatory response that may predispose envenomed victims to heart failure. These findings aligned with Kakumanu et al. [42], who observed cardiovascular collapse in *E. ocellatus* envenomed animals. However, we have established in our previous study that inflammatory responses in vital organs due to *E. ocellatus* envenomation was occasioned by the elevation of pro-inflammatory cytokines [29].

Enzymatic components of snake venom most especially those of viper venoms are known to activate, inhibit or liberate enzymes by destroying tissues and cellular organelles [31]. Venom of vipers are able to exhibits these effects due to the presence of enzymes such as metalloproteinase and phospholipase A2 which are proteolytic and lipolytic in nature respectively [43]. This explains the various histopathological defects noticed on the heart tissues of envenomed rats. In this study, *E. ocellatus* venom toxins induced attrition of cardiomyocyte fibers, mild epicardia hemorrhagic foci combined with tissue inflammation. The observed clinical pathologies further substantiated the biochemical results and the reduction noticed in the heart weight and index of the envenomed rats. These findings corroborated our previous reports on structural defects caused in heart tissues of rats envenomed with viper venoms [22,44]. Also, studies have reported that viper venoms have the potential to alter cardiac muscle structure and cause injury after envenomation [45]. The underlying mechanism of cardiotoxicity induced by *E. ocellatus* venom may remain unknown in this study. However, studies have elucidated that snake venom induced cardiotoxicity may be due to the presence of myotoxic PLA2 and other cardiotoxin(s) that are responsible for cellular necrosis and cytotoxicity by exhibiting a direct toxic effect on the cardiac tissues, myocardium, coagulation abnormalities and vasopasm [9,46].

Conclusion

This study has demonstrated that *E. ocellatus* venom has the intrinsic potentials to initiate cardiac toxicity through oxidative stress induction, pro-inflammatory reaction and structural alterations in the heart. These toxic effects may alter cardiac function and result in heart failure as observed in our findings. Although, timely intervention and treatments tends to improve responses in snakebite envenomed victims but assessment of cardiac condition should be considered important for effective management of snakebite patients.

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CRediT authorship contribution statement

Babafemi Siji Ajisebiola: Conceptualization, Methodology, Investigation, Supervision, Resources, Writing – original draft, Writing – review & editing. **Ayomikun Busayo Fawole:** Investigation, Methodology, Resources, Data curation. **Olubisi Esther Adeyi:** Formal analysis, Data curation, Writing – review & editing. **Akindele Oluwatosin Adeyi:** Conceptualization, Methodology, Project administration.

Declaration of interest

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.

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