



Artemia salina as an animal model for the preliminary evaluation of snake venom-induced toxicity

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ARTICLE INFO

Handling Editor: Dr. Raymond Norton

Keywords:

Artemia salina
Mice
Lethal concentration
Lethal dose
Necrosis

ABSTRACT

Lethality and cytotoxicity assays of snake venoms and their neutralization by antivenom require many mice for the experiments. Recent developments have prompted researchers to seek alternative strategies that minimize the use of mice in line with Russel and Burch's 3Rs philosophy (Replacement, Reduction, and Refinement). *Artemia salina* is an animal model widely used for toxicity screening. However, its use in snake venom toxicology is limited by a lack of data. The present study compared the toxicity of venoms from *Bitis arietans*, *Naja ashei*, and *Naja subfulva* using mice and *Artemia salina*. In the *Artemia salina* test at 24 h and the dermonecrotic test in mice, the toxicity of the venoms was in the order *Naja ashei* ~ *Naja subfulva* > *Bitis arietans*. In the lethality test in mice, the toxicity of the venoms was in the order *Naja subfulva* > *Naja ashei* > *Bitis arietans*. These findings suggest that the toxicity of the venoms in *Artemia salina* and the dermonecrotic bioassay in mice have a similar trend but differ from the lethality test in mice. Therefore, it may be relevant to further explore the *Artemia salina* bioassay as a potential surrogate test of dermonecrosis in mice. Studies with more venoms may be needed to establish the correlation between the *Artemia salina* bioassay and the dermonecrotic assay in mice.

1. Introduction

Antivenom efficacy is primarily evaluated in mice using the neutralization of lethality assay (WHO, 2016). This is a preclinical test that is routinely carried out by antivenom manufacturers in fulfillment of regulatory requirements (Gutiérrez et al., 2013, 2021). Investigating the capacity of antivenoms to neutralize other toxic effects of venom e.g. dermonecrosis may also be important (Gutiérrez et al., 2021; 2013; WHO, 2016). Towards this end, the World Health Organization (WHO) recommends the *in vivo* minimum necrotizing dose (MND) assay in mice (WHO, 2016).

Assays on the toxicities of venom e.g. lethality and dermonecrosis and their neutralization by antivenom require many mice for the experiments, which are costly and labor-intensive. Criticism by animal

welfare groups is also rife (da Silva et al., 2015; Kakanj et al., 2015; Kerckamp et al., 2018; Stransky et al., 2018). Therefore, researchers are motivated to seek alternative techniques which may significantly minimize the use of or eliminate the need to use mice (Calvete et al., 2016; Barbosa et al., 1995; Chacón et al., 2015; De souza et al., 2015; Khaing et al., 2018; Oguiura et al., 2014; Pornmuttakun and Ratanabangkoon, 2014; Rial et al., 2006; Rungsiwongse and Ratanabangkoon, 1991; Segura et al., 2010; Theakston and Reid, 1979).

The lethality assay in brine shrimp (*Artemia salina*) is rapid and requires minimal resources (Hamidi et al., 2014). It has many applications in toxicology (Barahona and Sanchez-Fortun, 1999; Gadir, 2012; Hernández-Matehuala et al., 2015; Kerster and Schaeffer, 1983; Mirzaei and Mirzaei, 2013; Mwangi et al., 2015; Okumu et al., 2020; Hamidi et al., 2014; Rajabi et al., 2015, 2012). However, its suitability as a

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<https://doi.org/10.1016/j.toxcx.2021.100082>

Received 16 June 2021; Received in revised form 12 August 2021; Accepted 16 August 2021

Available online 19 August 2021

2590-1710/© 2021 The Author(s).

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replacement or surrogate test to the more technically and ethically challenging lethality or necrotizing assays is limited by a lack of data. This study compared the *Artemia salina* lethality assay with the lethality and necrotizing assays in mice using venom from some snakes of medical importance in Sub-Saharan Africa namely *Bitis arietans* (Puff adder), *Naja ashei* (large brown spitting cobra), and *Naja subfulva* (Eastern Forest Cobra).

2. Materials and methods

2.1. Ethical considerations

Ethical approval was obtained from the institutional Biosafety, Animal Care, and Use Committee REF BAUEC/2019/220.

2.2. Snake venom

Venom was collected from snakes (*Bitis arietans*, *Naja ashei*, and *Naja subfulva*) which are maintained in Kenya (Bioken Snake Farm, Malindi). Freshly collected venom from each snake species was pooled, snap frozen, lyophilized, and stored at -20°C . Freeze-dried venom samples were reconstituted in phosphate-buffered saline at the time of use.

2.3. *Artemia salina* (brine shrimp) lethality assay

Artemia salina (brine shrimp) eggs were commercially sourced (Batch number; X001M8M5IZ) and hatched in a trough using marine salt solution (MSS; 38.5% w/v) over 48 h. *Artemia* nauplii/larvae were transferred from the hatching trough to 5 mL sample vials. Venom aliquots (5, 50, and 500 μL) were pipetted from venom stock solutions (5 mg/mL) into the vials which contained brine shrimp. The contents were made up to the mark with MSS resulting in 10, 100, and 1000 $\mu\text{g}/\text{mL}$ venom concentrations (Meyer et al., 1982). The nauplii that died were counted after 24 and 72 h. Experiments were carried out in quintuples of 10 nauplii per vial. Mortality was analyzed by probit analysis and the results were expressed as LC_{50} : Median lethal concentration (Bliss, 1935; Finney, 1952). In cases where there were deaths in the control group (phosphate-buffered saline), the data were corrected using the formula by Abbot (Abbott, 1925).

$$\% \text{Death} = \frac{\text{Test} - \text{Control}}{100 - \text{Control}} \times 100$$

2.4. The median lethal dose (LD_{50}) assay in mice

Eighty mice weighing 18–20 g were randomly assigned to 16 groups of 5 mice each. Graded doses of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venoms were prepared in phosphate-buffered saline and administered to 75 mice (WHO, 2016). Five mice served as control and received phosphate-buffered saline only. Mortality was recorded after 24 h and the least dose of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venom which was responsible for 50% mortality was determined by Probit analysis (Finney, 1952).

2.5. The minimum necrotizing dose (MND) assay in mice

Eighty mice weighing 18–20 g were shaved (Theakston and Reid, 1983; WHO, 2016). Solutions (50 μL) containing graded doses of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venoms were injected intradermally in the shaved skin of mice (Theakston and Reid, 1983; WHO, 2016). After 3 days, the mice were humanely sacrificed and the skin was carefully removed. The diameter of snake venom-induced necrotic lesions on the inner side of the skin was measured using a digital Vernier caliper (Theakston and Reid, 1983; WHO, 2016). A plot of the mean lesion diameter against the venom doses was used to estimate the minimum necrotizing dose (MND) which was defined as the dose of

venom which corresponded to a 5-mm necrotic lesion (Theakston and Reid, 1983; WHO, 2016). A negative control group received phosphate-buffered saline only.

2.6. Statistical analysis

The *Artemia salina* LC_{50} values of the venoms were determined by probit analysis (Bliss, 1935; Gaddum, 1948). The minimum necrotizing dose was defined as the least amount of venom which when injected intradermally produced a necrotic lesion of 5 mm diameter. It was estimated by plotting mean lesion diameter against venom dose and reading off the dose which corresponded to a 5-mm diameter (Theakston and Reid, 1983; WHO, 2016).

3. Results

Information on the gender, size, location of capture, and reference number of the snakes used in venom extraction is shown in Table S1. Adult, male and female snakes were collected from Watamu, Kizingo, the Arabuko Sokoke Forest, Kilifi, Kakamega, Busia, and Nandi. Table S1.

The mortalities of *Artemia salina* larvae treated with phosphate-buffered saline only (negative control) are summarized in Table S2. Some mortality was observed in controls at 72 h. *Bitis arietans*, *Naja ashei*, and *Naja subfulva* snake venom-induced mortality in *Artemia salina* is summarized in Tables S3, S4, and S5 respectively. Generally, there were dose and time-dependent increases in the venom-induced mortalities of *Artemia salina*. See Tables S3, S4, and S5.

According to the data of Table 1, the toxicity of snake venoms in *Artemia salina* after 24 h was in the order *Naja ashei* ~ *Naja subfulva* > *Bitis arietans*. Table 1. The toxicity (lethality) of the venoms in mice after 24 h was in the order *Naja subfulva* > *Naja ashei* > *Bitis arietans*. Table 1. The necrotizing activity of the venoms in mice after 72 h of exposure was in the order *Naja subfulva* ~ *Naja ashei* > *Bitis arietans*. Table 1.

4. Discussion

The search for alternatives to the mouse lethality/toxicity assays in snake venom research has been a subject of fascination for many researchers past and present. As far back as 1907, Albert Calmette described a relationship between toxicity in mice and *in vitro* proteolysis and hemotoxicity (Calmette, 1907). Russel and Burch's 3Rs philosophy (Reduction, Replacement, and Refinement) limits the number of mice used in experimental assays and is widely celebrated in the snake venom toxinology community (Russel and Burch 1959). In line with this philosophy, the present study presents baseline data on the applicability of an alternative animal model for the preliminary evaluation of snake venom-induced toxicity.

Lethality is an acute response to venom while dermonecrosis takes some time to unravel (i.e. up to 72 h) (WHO, 2016). In the present study, murine LD_{50} and *Artemia salina* bioassays were compared at different times i.e. the toxicity in *Artemia salina* (24 and 72 h) and the toxicity in mice (24 and 72 h). A good relationship was observed between the 24 h *Artemia salina* bioassay and the 72-h dermonecrosis bioassay. In other words, the *Artemia salina* test does not necessarily have to be done at 72 h. However, a major problem of running the *Artemia salina* test for an extended period (72 h) was the high number of deaths in the negative control (phosphate-buffered saline) samples. Therefore, based on these results, the best time to judge the lethality of *Artemia salina* would be after 24 h.

The mortality trends of the venom in *Artemia salina* were inconsistent with mortality in mice. On the strength of these findings, the *Artemia salina* model does not appear to be suitable for evaluating neurotoxic venoms. It could be argued that the neurotoxins of elapid venom (*Naja subfulva*), most of which act on nicotinic acetylcholine receptors of muscle cells (Kukhtina et al., 2000), do not act on the synapsis of *Artemia*

Table 1Comparison of the toxicity of *Bitis arietans*, *Naja ashei* and *Naja subfulva* snake venoms in *Artemia salina* and in mice.

Snake species	Toxicity in <i>Artemia salina</i> ^a		Toxicity in mice	
	LC ₅₀ (24 h)	LC ₅₀ (72 h)	LD ₅₀ (24 h) ^b	MND (72 h) ^c
<i>Bitis arietans</i>	410.47 (228.21–729.14)	57.36 (26.50–125.47)	9.24 (7.50–11.38)	112.92
<i>Naja ashei</i>	41.28	5.40	3.02 (2.45–3.72)	36.12
<i>Naja subfulva</i>	47.54	12.06	0.55 (0.49–0.63)	31.48

^a Expressed as Median Lethal Concentration (LC₅₀) in µg/mL, with 95% confidence limits in parentheses.

^b Expressed as Median Lethal Dose (LD₅₀) in µg/g, with 95% confidence limits in parentheses.

^c Expressed as Minimum Necrotizing Dose (MND) in µg.

salina. However, the observation that high doses of the venoms were able to kill the *Artemia salina* suggests that there may be some neurotoxic effect realized. Perhaps these neurotoxins cannot readily penetrate the tissues of *Artemia salina*.

It was interesting to note that the cytotoxic venom (*Naja ashei*) was more toxic to *Artemia salina* at 72 h than the neurotoxic *Naja subfulva* venom. This could imply that the cytotoxins (either 3FTxs or PLA₂s) in *Naja ashei* venom (Hus et al., 2018) have the capacity to damage membranes of the *Artemia salina*. The findings of this study when taken together with the advantages of the *Artemia salina* assay (affordability, rapidity, simplicity, convenience, and robustness) and its utility in antivenom efficacy testing (Okumu et al., 2020) make a strong case for its use as an alternative to the more technically challenging, and expensive minimum necrotizing dose assay in mice. However, despite the many positive aspects of the assay, there are some drawbacks associated with the use of *Artemia salina*. First, it does not provide adequate information on the mechanism of cytotoxicity of the test substance (Hamidi et al., 2014). Secondly, the hatching of the *Artemia nauplii* requires a container that should be large enough to hold an air pump for equal distribution of oxygen (Vanhaecke et al., 1981). Moreover, 48-h old nauplii (2nd to 3rd instar stage) are more sensitive to test compounds (Vanhaecke et al., 1981). Thus many of the nauplii which hatch beyond the 48-h incubation period may not be suitable for experimental assays. Variations in pH and temperature may also affect the hatching process of the nauplii (Sorgeloos et al., 1978).

5. Conclusion

These findings suggest that the toxicity of the venoms in *Artemia salina* and the dermonecrotic bioassay in mice have a similar trend. Therefore, it may be relevant to further explore the *Artemia salina* bioassay along the lines of the 3Rs concept aimed at reducing the use of mice in snake venom research. Studies with a larger number of venoms are warranted to determine the correlation between the *Artemia salina* bioassay and the dermonecrotic assay in mice.

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.

Acknowledgment

The authors would like to acknowledge Ms. Clare Taylor and Boniface Momanyi of Bioken snake farm (Watamu, Kenya) for providing the venom samples. We would also like to thank Mr. Joseph Nderitu and Mr. Kenneth Maloba of the Department of Public Health, Pharmacology and Toxicology, the University of Nairobi for their assistance in conducting some of the assays. The graphical abstract was designed using Biorender.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2021.100082>.

[org/10.1016/j.toxcx.2021.100082](https://doi.org/10.1016/j.toxcx.2021.100082).

Author contributions

Conceptualization: MO, FO, and JG; data curation: all authors; formal analysis: MO; investigation: all authors; methodology: MO, FO, and MJ.; project administration: MO, JG, and FO, resources; all authors; software: MO; supervision: JM, JG, PM, and VM; validation: FO, JM, JG, PM, VM, and MJ; visualization: MO, and MJ; writing original draft: MO and FO; writing review and editing: all authors. All authors read and approved the final manuscript.

Ethical considerations

This study was approved by the Biosafety, Animal Care, and Use Committee of the Faculty of Veterinary Medicine, University of Nairobi. REF BAUEC/2019/220 which was issued on April 24, 2019. All the used cell lines were disposed of per the Kenya Medical Research Institute (KEMRI)-University of Nairobi (UoN) protocols.

Consent for publication

Not applicable.

Funding

This study was funded by the National Research Fund (REF NRF/Ph. D./02/158).

Abbreviations

MND Minimum necrotizing dose; WHO: World Health Organization; BAUEC: Biosafety Animal Use and Ethics Committee; KEMRI: Kenya Medical Research Institute; UoN: University of Nairobi; LC₅₀: the lethal concentration responsible for 50% mortality in *Artemia salina*; LD₅₀: the median lethal dose responsible for 50% mortality in mice.

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