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Toxicity of scorpion venom in chick embryo and mealworm assay depending on the use of the soluble fraction versus the whole venom

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ABSTRACT

The LD_{50} is an important metric for venom studies and antivenom development. It has been shown that several variables in the protocol influence the LD_{50} value obtained, such as venom source, extraction and treatment and administration route. These inconsistencies reduce the utility of the results of these test for comparative studies. In scorpion venom LD_{50} assays, often only the soluble fraction of the venom is used, whereas other studies use the whole venom. We here tested the toxicity of the soluble fraction in isolation, and of the whole venom in two different systems: chick embryos and mealworms *Tenebrio molitor*. Ten microliters of venom solutions from *Hadrurus arizonensis*, *Leiurus quinquestriatus*, *Androctonus australis*, *Grosphus grandidieri* and *Heterometrus laoticus* were applied to five day old chicken embryos at stage 25–27. Our results showed no significant differences between the LD_{50} based on the whole venom versus that of only the soluble fraction and in the chicken embryo assay in four of the five scorpion species tested. *H. laoticus* however, showed a significantly lower LD_{50} value for the whole venom than the soluble fraction. In assays on mealworms however, this pattern was not seen. Nonetheless, caution may be warranted when using LD_{50} values obtained from only the soluble fraction. The LD_{50} values of the five species in this study, based on the chicken embryo assay, showed good correlation with values from the literature based on mouse studies. This suggests that the chick embryo assay may be an economic alternative to rodent assays for scorpion LD_{50} studies.

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1. Introduction

Many animal venoms are a complex mixture of different compound classes including large proteins, peptides, small molecules and salts. Venom peptides are often exaptations of normal metabolic peptides (Vonk et al., 2013), which were duplicated and subsequently adapted to their role in disrupting the target organism's physiology (Cao et al., 2013; Fry et al., 2009). The high level of biological activity in venoms has been suggested to evolve in an arms-race of the source organism in defense against, or for incapacitation of, a specific class of organisms (Barlow et al., 2009). In addition to toxins directed against their arthropod prey (e.g. Arnon et al., 2005; D'Suze et al., 2004; De Dianous et al., 1987; García et al., 1997; Pimenta et al., 2001; Selisko et al., 1996), venoms from scorpions may additionally contain toxins against vertebrate predators (Ghane et al., 2008; Lazarovici and Zlotkin, 1982; Luo et al., 1997; Possani et al., 1999). It also has antibacterial properties (Remijsen et al., 2010). As in snake venom (Daltry et al.,

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1996), the efficacy of scorpion venoms differs highly between species and populations (Devaux et al., 2004; El Ayeb and Rochat, 1985; Newton et al., 2007; Smertenko et al., 2001).

One of the most basic metrics of the efficacy of venom is the LD_{50}, or the dose at which half the test animals exposed to the toxin die. The LD_{50} is used to estimate the toxicity of venom in a certain class of target organisms, and is important in the development of antisera (Krifi et al., 1998). Assessment the LD_{50} of scorpion venom has traditionally been conducted on laboratory mice as a vertebrate target. LD_{50} protocols in the literature are rarely consistent, varying in venom source, extraction and treatment, as well as administration route (*oral*, *intracutaneous*, *intravenous*, *intraperitoneal*). These differences in protocol can make it hard to compare venom efficacies between studies, reducing the utility of the results of these tests for comparative studies. This is especially undesirable as there are ethical concerns about using mice for LD_{50} testing of venom, the assays are costly, as well as require up to several thousand scorpions to be milked or sacrificed (Krifi et al., 1998). For these reasons, LD_{50} values have been published for relatively few of the almost 2000 species of scorpions. It has been shown that the method of venom extraction and administration can also influence the LD_{50} considerably (Krifi et al., 1998). It was also found that LD_{50} levels differ considerably between commercial venoms, those made from the telsons of euthanized scorpions, and those acquired by electrical stimulation, with the latter having the lowest value (Ozkan and Filazi, 2004).

Since most medically relevant species of scorpions are a threat to human health due to their possession of small neurotoxic peptides, most LD_{50} assessments have concentrated on these smaller molecules. Many LD_{50} studies therefore follow Miranda et al. (1970) in using water extraction, in some cases followed by acetone fractionation, to separate the smaller active molecules from the mucoproteins considered to make up the insoluble fraction of the venom. In this often used protocol, the aqueous venom solution is centrifuged and only the supernatant is used (Calderon-Aranda et al., 1993; Deshpande et al., 2005; Ismail et al., 1992, 1974, 1973; Krifi et al., 1996; Manzoli-Palma et al., 2003; Ozkan and Carhan, 2008). The insoluble fraction, which gives scorpion venom its characteristic milky appearance, was therefore not assayed in those studies. In other studies however, the whole venom is used (Hafny et al., 2002; Tarasiuk et al., 1998; Tiwari and Deshpande, 1993; Zlotkin et al., 1971).

In this study, we explore the effect of removing the insoluble fraction of venom by water extraction and centrifugation on the *in vivo* efficacy of scorpion venoms by comparing the LD_{50} of whole homogenized scorpion venom with that of the soluble fraction alone.

2. Materials and methods

2.1. Venom preparation

Venom from five species was used: *Hadrurus arizonensis*, *Leiurus quinquestriatus*, *Androctonus australis*, *Grosphus grandidieri* and *Heterometrus laoticus*. In the remainder of the article, these species will be referred to by their generic names. We extracted venom from live scorpions by applying a square wave voltage to the metasoma, alternating between 0 and 37 V, at a rate of 20–50 Hz. Saline solution was added at the contact points to facilitate electrical conductance. The telson itself was not part of the circuit, thereby avoiding any changes to the venom due to the applied voltage. Venom was collected in low protein-binding 2 ml tubes (Simport, Beloeil, Canada), snap-frozen in liquid nitrogen, and stored at −20 °C until they were lyophilized. Venoms were separated in different batches, and prepared by different methods; whole venom preparations were made by dissolving lyophilized venom in Hank's balanced salt solution, and vortexing or shaking with glass beads at 30 Hz until homogeneously suspended. Soluble fraction preparations were made by dissolving the lyophilized venom in ultrapure water (Milli-Q, Millipore, Billerica MA, USA), vortexing, and centrifuging at 4000 g for 5 min. The supernatant was then transferred to a low protein-binding Eppendorf tube (Eppendorf, Hamburg, Germany), lyophilized, and later dissolved in Hanks' salt solution by vortexing until fully dissolved.

2.2. In vivo assays

Venom preparations were tested on 5 day old chicken embryos as a vertebrate model, and injected into mealworm (*Tenebrio molitor*) larvae as an arthropod model. In both the chicken embryo and mealworm assays, range-finding was conducted with a logarithmic concentration series; this was then followed by a geometric concentration series lying within the effective concentration range.

2.2.1. Chick embryos

Following Sells (1998, 1997, 2001; 2003), we used fertilized chicken eggs incubated in a humidified, forced-draft incubator with stationary shelves at 38 °C for 5 days. A sample of 15 eggs and embryos (Stage 25–27, untreated) were weighed. Eggs weighed 60.3 g ± 4.26 g and embryos 0.23 ± 0.056 g. Contrary to the method of Sells, the contents of the eggs were not transplanted out of the eggshell, but a small opening was made to allow access. The vitelline membrane and amnion were opened to allow access to the embryo. Venom solutions were applied by pipetting 10 μl directly onto the embryo, near the yolk sac attachment. The window was then closed with a small piece of Sellotape®, and the eggs returned to the incubator for another two days. Between 10 and 12 eggs were used for each venom concentration. Mortality was assessed by candling the eggs at 24 h and 48 h after venom application. Eggs with no distinguishable vasculature were scored as dead. The accuracy of this fast assessment method by candling was confirmed by opening a subset of the eggs tested and examining the embryo. Only the mortality at 24 h after application of the venom was used in calculating the LD_{50}. Mortality was corrected for mortality in the control group (Schneider-Orelli, 1947). Since all experiments were carried out under the same conditions in the same incubator, corrected mortality numbers from successive experiments were combined in the LD_{50} calculation.

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