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Spine-bellied sea snake (*Hydrophis curtus*) venom shows greater skeletal myotoxicity compared with cardiac myotoxicity

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ABSTRACT

For the first time the impedance-based xCELLigence real-time cell analysis system was used to measure the myotoxicity of sea snake venom. With a focus on the spine-bellied sea snake (*Hydrophis curtus*), the venom of four sea snake species and three terrestrial snake species were compared for myotoxicity against a human skeletal muscle cell line (HSkMC). *Hydrophis curtus* venom was also tested on a human cardiac muscle cell line (HCM). Surprisingly, all four sea snake venoms tested on HSkMC produced an initial 100–280% rise in xCELLigence cell index that peaked within the first two hours before falling. The cell index rise of *H. curtus* venom was correlated with the WST-1 cell proliferation assay, which demonstrated an increase in mitochondrial metabolism. The myotoxicity of *H. curtus* was 4.7–8.2 fold less potent than the other sea snakes tested, the Australian beaked sea snake (*Hydrophis zweifeli*), the elegant sea snake (*Hydrophis elegans*) and the olive sea snake (*Aipysurus laevis*). If our cell-based results translate to *H. curtus* envenomations, this implies that *H. curtus* would be less myotoxic than the other three. Yet the myotoxicity of *H. curtus* venom to cardiac muscle cells was nine times weaker than for skeletal muscle cells, providing evidence that the venom has a selective effect on skeletal muscle cells. This evidence, combined with the slow-acting nature of the venom, supports a digestive role for sea snake myotoxins.

1. Introduction

The viviparous “true” sea snakes (Elapidae: Hydrophiinae: Hydrophinii) consist of at least 60 species, most of which fall into one of two major genera (Sanders et al., 2013). The *Aipysurus* genus comprises 10 species, while the larger *Hydrophis* group contains at least 49 species (Sanders et al., 2013). Sea snake species are found in the shallow subtropical and tropical marine waters of the western Pacific, Indian Oceans and the Persian Gulf (Rasmussen et al., 2011; White, 1995). The pelagic *Hydrophis platurus* is also found along the east coast of Africa and in the tropical waters of the western Americas (Rasmussen et al., 2011; White, 1995).

Many sea snake species are highly venomous to humans. *Hydrophis schistosus*, synonymous with *Enhydrina schistosa*, has been responsible for serious and fatal envenomations (Kularatne et al., 2014; Reid, 1975a, b). For example, in Reid's epidemiological investigations of sea snake bite cases in north-west Malaya, he found that *H. schistosus* was responsible for over half of the 101 cases admitted to hospital, including seven of the eight lethal bites recorded (Reid, 1975b). Several other species have caused severe or deadly envenomations, including the annulated sea snake (*Hydrophis cyanocinctus*) (Reid, 1975a), the spine-bellied sea snake (*Hydrophis curtus*, synonymous with *Lapemis curtus, Lapemis hardwickii* and *Hydrophis hardwickii*) (Vijayaraghavan and Ganesh, 2015; Warrell, 1994) and Stokes' sea snake (*Hydrophis stokesii*, synonymous with *Astrotia stokesii*) (Mercer et al., 1981). However, in many cases the species remains unidentified (Reid, 1975a; Warrell, 1994).

There is no current estimate for the number of sea snake envenomations that occur worldwide per year. By extrapolating data collected in Malaya by Reid in the 1950s–1970s (Reid, 1975a), White (1995) suggested the yearly total could be between 15,000 and 75,000 envenomations, with approximately 10% of those being fatal. Reid's epidemiological study found that fishermen using

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traditional methods are at greatest risk (80 out of 101 cases), particularly when handling nets or sorting fish (Reid, 1975a, b). Bathers were the next at-risk group, comprising 14 of the cases, all of which occurred after treading on a sea snake (Reid, 1975a, b). About 20% of sea snake envenomations are serious (Reid, 1975b). Without prompt treatment patients experience systemic myolysis, neuromuscular paralysis or a combination of both (Reid, 1975a; White, 1995; White, 2013). The clinical presentation reflects the two main toxin families found in sea snake venom, myotoxic phospholipase A_2 (PLA_2) and the neurotoxic three-finger toxins (3FTx) (Calvete et al., 2012; Laustsen et al., 2015; Lomonte et al., 2014; Tan et al., 2015a). Myolysis is considered to be the most prevalent clinical presentation (Reid, 1961; White, 1995, 2013). The onset of symptoms is rarely immediate, developing between 30 min and 3.5 h post-bite (Reid, 1975a). An onset before 2 h often predicts severe envenomation (Reid, 1975a; White, 1995).

CSL Limited sea snake antivenom has been shown to successfully neutralise the venoms of a wide range of sea snake species, including those from the *Aipysurus* and *Hydrophis* genera (Chetty et al., 2004; Laustsen et al., 2015; Lomonte et al., 2014). Unfortunately, many envenomations occur in areas where the antivenom is not readily available due to lack of access and/or cost (Tan et al., 2015b). In Malaysia, for example, sea snake antivenom is approximately 3000 US dollars per vial (Tan et al., 2015b) and more than one vial may be needed in a severe envenomation (White, 2013). The widespread distribution of sea snakes, along with their potent venom, makes serious envenomation likely to occur. For these reasons, combined with the difficulties in accessing treatment where sea snake envenomation is common, it is essential to understand how the venom functions. This knowledge could lead to improvements in first aid and clinical management, and may allow the development of an effective yet inexpensive alternative treatment.

Due to the apparent dominance of myolysis in sea snake bites, this study aims to add to knowledge of sea snake venom by specifically examining its myolytic effects. Biopsy and autopsy evidence on humans and experimental evidence on mice shows that sea snake venom primarily affects the skeletal muscles, with little sign of cardiovascular damage (Brook et al., 1987; Gutiérrez and Ownby, 2003; Marsden and Reid, 1961). We considered two possible explanations: (1) the venom might affect skeletal muscles more extensively because it comes into contact with skeletal muscles first, due to the route of envenomation, and/or (2) the venom could act selectively on skeletal muscles. To explore which of these possibilities is most probable the high-throughput xCELLigence real-time cell analysis system was employed to test the effects of *H. curtus* venom on a human skeletal muscle cell line and a human cardiac muscle cell line. xCELLigence has been successfully used to model the effects of box jellyfish (*Chironex fleckeri*) whole venom and isolated toxins on human cell lines (Andreosso et al., 2014; Chaousis et al., 2014; Pereira and Seymour, 2013; Saggiomo and Seymour, 2012), with results indicating a strong correlation with alternative cell viability assays (Chaousis et al., 2014). The current research suggests xCELLigence may be a useful tool to measure myotoxicity in sea snake venom in real time. Furthermore, the xCELLigence cell index output against skeletal muscle cells was noted to mimic the slow progression of muscle breakdown symptoms observed in human envenomations.

2. Materials and methods

2.1. Venom samples

Hydrophis curtus venom was collected from 19 wild individuals caught off Hey Point, Weipa (12°43′46.6″S 141°53′34.4″E) in the Gulf of Carpentaria, Australia in June 2016. Individuals were all subadults, where 'subadult' is defined as an individual <400 mm in length. All *H. curtus* venom samples were stored separately in a liquid nitrogen dewar. Venom samples were collected from three individual elegant sea snakes (*Hydrophis elegans*) and a single Australian beaked sea snake (*Hydrophis zweifeli*) in January 2016, also from Hey Point, Weipa. Venom from an olive sea snake (*Aipysurus laevis*) was collected in 2010 from Pixie Pinnacle at the southern end of Ribbon Reef 10 (14°55′39.5″S 145°41′43.2″E), Great Barrier Reef, Australia. Venoms from three Australian terrestrial snakes, the mulga (*Pseudechis australis*), inland taipan (*Oxyuranus microlepidotus*) and desert death adder (*Acanthophis pyrrhus*), were sourced from Cairns Tropical Zoo, Cairns, Australia in 2008. All venoms aside from the *H. curtus* samples were lyophilised and stored at −80 °C.

2.2. Pooling H. curtus venom samples

Ten *H. curtus* venom samples were chosen at random. Each was mixed with 500 μL of Milli-Q water, then all were pooled together. Pooled venom was separated into 100 μL aliquots, lyophilised and stored at −80 °C.

2.3. The xCELLigence real-time cell analysis system

The xCELLigence real-time cell analysis (RTCA) system (single plate, ACEA Biosciences) uses a 96-E-well plate with gold microelectrodes set into the bases of the wells, which are seeded with cells (Urcan et al., 2010). The seeded plate is placed into the RTCA instrument, which applies an electric potential (approximately 20 mV) across the negative and positive electrodes through the cell culture medium (Urcan et al., 2010). If cells are attached to the base of a well, electron flow is impeded; as cells detach, such as when a cytotoxic substance is added, electron flow increases (Urcan et al., 2010). The degree of impedance is monitored over time by the integrated RTCA 2.0 software and reported as a unitless parameter called the cell index, where decreasing cell index indicates increasing electron flow and thus decreasing cell viability (Urcan et al., 2010).

2.4. Cell culturing, plate preparation and xCELLigence monitoring

A human skeletal muscle cell line (HSkMC) and a human cardiac muscle cell line (HCM) from ScienCell were cultured in HSkMC and HCM medium with 5% foetal bovine serum at 37 °C with 5% CO_2 in 75 cm^3 monolayer flasks according to the manufacturer's protocol. An average of 5000 cells in 150 μL of the appropriate medium were seeded in each well of a 96-E-well plate (ACEA Biosciences) and incubated for 24 h at 37 °C with 5% CO_2 before venom addition. For all xCELLigence experiments, concentrated samples and controls were prepared on ice in a separate 96-well plate at 8.5 times the in-well concentration. Dulbecco's phosphate-buffered saline (DPBS; Gibco) was used for mixing venom and as a negative control with three to six replicates. Each concentrated sample or control was added at 20 μL to each well of the xCELLigence E-plate (combined total volume 170 μL). Immediately after venom addition cell status was monitored continuously with the RTCA 2.0 software for a minimum of 48 h at the following intervals: 100 sweeps every 15 s, 100 sweeps every 2 min, 100 sweeps every 15 min and hourly for the remainder. Protein concentrations used in the xCELLigence and WST-1 experiments were determined using the Bradford-Lowry protein assay (Bradford, 1976; Sedmak and Grossberg, 1977) according to the manufacturer's protocol (Thermo Scientific).