Heat deactivation of the stonefish *Synanceia horrida* venom – implications for first-aid management

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Abstract
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Objectives: To investigate the effects of temperature and hot water immersion time on neutralising venom lethality of the Australian estuarine stonefish (*Synanceia horrida*).
Design: Depths of the spines were measured while venom was extracted from *S. horrida* individuals. The venom was then exposed to temperatures of 4^{O}C, 37.0^{O}C, 40.1^{O}C, 42.3^{O}C, 45.0^{O}C, 47.7^{O}C, 55.2^{O}C, and 60.0^{O}C for either five or 20 minutes incubation periods. Venom samples were added to cultured human cardiomyocytes and cell viability curves were produced using the ACEA's xCELLigence real-time cell monitoring system.
Main outcome measures: Determination of venom lethality on cardiomyocytes at a range of temperatures.
Results: The average depth of the spine required to go into a victims' flesh before the venom gland compressed and expelled venom was 18 mm. Cardiomyocytes exposed to heat-treated venom for five minutes required higher temperatures to neutralise 99% of the venom, namely 44.6^{O}C in comparison to 42.1^{O}C with an incubation time of 20 minutes.
Conclusion: This study supports the use of hot water immersion therapy in the treatment of *S. horrida* stings. It is suggested that due to the depth of the puncture wound longer incubation times should be sought to allow heat to penetrate the deeper portions of the dermis and effectively begin venom deactivation.

Introduction

Hot water immersion (HWI) therapy has been the principal first-aid treatment employed for the alleviation of pain in fish envenomation injuries throughout the past two centuries.[1] HWI treatment has shown to be effective across a large suite of piscine families, perhaps the most notable being the highly venomous stonefishes (*Synanceiidae* family).[2–6] Despite the duration of time HWI therapy has been practiced and the fact that it appears to be effective, no detailed studies have been performed to analyze how temperature and exposure time influence the lethal behaviour of fish venom.

Envenomation from stonefishes occurs when force is applied to the integument sheath that encases the dorsal spine of the animal, along with the compression of the dual venom sacs on either side of the spine. The venom sacs can be a third to a half of the length of the spine and subsequently excrete venom along a venom duct in the spine and into the contacting body.[7,8] Despite injuries being minor for the most part, severe scenarios have been documented. The symptoms of envenomation encompass immediate and radiating pain, appreciable local morbidity and paralysis, gross oedema, headache and, in severe cases, hypotension, bradycardia, arrhythmia, heart failure and death.[9–12]

Studies suggest that owing to the proteinaceous nature of the venom,[13–20] heating the solution will cause deactivation of the protein components and thus reduce the venom activity. It has been observed at temperatures of 50^{O}C that venom toxicity is neutralized;[20] however, treating patients with water at this temperature could result in skin burns and tissue necrosis.[21] Current treatment protocols suggest that the victim be treated with HWI around 42–45^{O}C for 30 to 90 minutes (min).[22,23] This is a generalised procedure done for stings or stabs produced by stingrays, starfish, sea urchins, weeverfish, scorpionfish and stonefish.[11] To date, no activity range for stonefish venom and its relationship with heat have been produced, thus no protocols specifically exist for stonefish envenomations.

Consequently, this study aims to investigate the thermo-labile behaviour of *Synanceia horrida* venom. More specifically, we explored temperatures that provide therapeutic benefit for treating stonefish envenomations achieved by examining the effect of venom toxicity with varying temperatures and heat exposure times.

Methods

Venom was collected from mature *S. horrida* housed at the James Cook University Cairns Campus research aquarium

Figure 1
The Australian estuarine stonefish (*Synanceia horrida*)

Figure 2
The intact exposed venom apparatus (dorsal spine and paired venom glands) of the Australian estuarine stonefish (*Synanceia horrida*)

Figure 3
High resolution photographs of the dorsal spine of the Australian estuarine stonefish (*Synanceia horrida*) being compressed with a rubber strip: A – downwards pressure first being applied to spine; B – integument sheath surrounding the venom gland has been compressed and venom is being released from the hollow duct through the spine; C – venom gland is compressed and venom is still being released under pressure; D – venom gland fully compressed and has been emptied of its volume

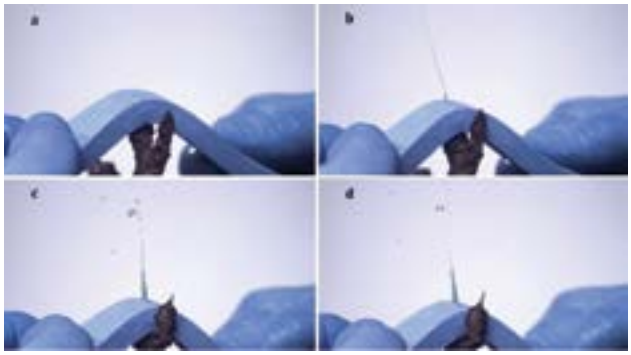
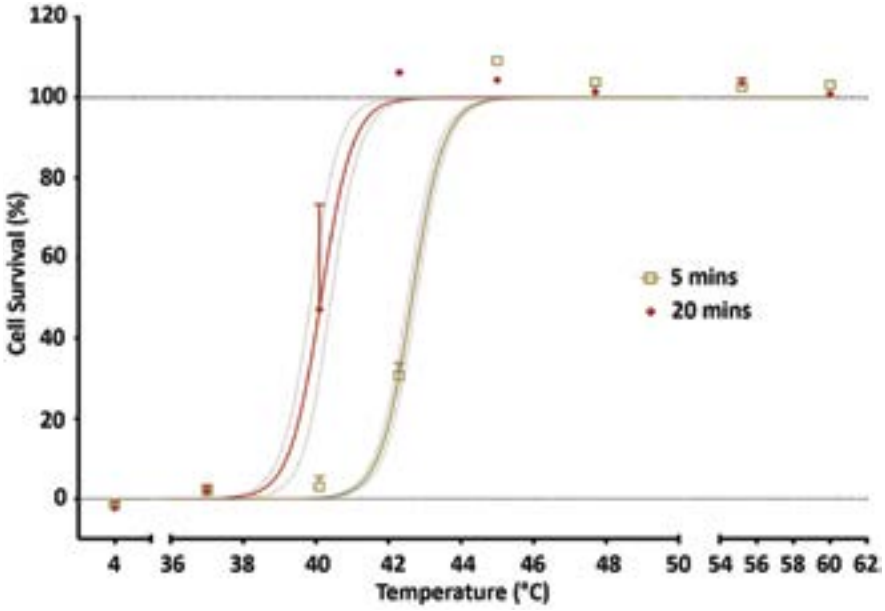


Figure 4
Cell survival of human cardiomyocytes when exposed to *Synanceia horrida* venom at different temperatures for two different incubation times (5 and 20 minutes); error bars represent standard deviation in replicates



facilities (Figure 1). Venom was aspirated from the dorsal spine glands using a 29 g half-inch needle inserted through the skin membrane into the venom gland (Figure 2). Samples were frozen at -80°C, lyophilized and returned to a -80°C freezer setting for storage until use. Venom was rehydrated with Dulbecco's phosphate buffered saline and centrifuged at 22,402 g for 2 min. The centrifuged supernatant was cleaned using a 0.22 µm filter and protein concentration determined using the Bradford Lowry protein assay.[24]

Subsequently, venom samples were heated to 4°C, 37.0°C, 40.1°C, 42.3°C, 45.0°C, 47.7°C, 55.2°C and 60.0°C for incubation times of 5 min and 20 min. Samples were then removed from the heating plate before being returned to an ice bath (4.0°C) before testing. ACEA's xCELLigence system was used to assess the cytotoxicity of the venom on human cells *in vitro*. Samples were aliquoted into individual wells on the xCELLigence E-plate seeded with 5,000 human cardiomyocytes per well. A total of three replicates for each temperature and exposure time were used, with controls consisting of four replicates of unheated venom rehydrated with Dulbecco's phosphate buffered saline solution.

To understand the depth at which the integument sheath is broken and forces venom to be expelled through the spine, the first dorsal spine length was measured from spine tip to the beginning of the venom sac in individual animals. To achieve this measurement, a rubber sheet was compressed down onto the venom sac and the distance from the spine tip was recorded (Figure 3).

STATISTICAL ANALYSIS

Data were analyzed using an analysis of covariance for temperature as the independent variable, cell viability as the dependent variable and incubation time as the co-variate, with curves fitted using a variable hill slope (-1). The 0.01% inhibition concentration, Chi-square goodness of fit test (extra sum-of-squares F-test) and graphs were generated with GraphPad Prism v 6.0 (GraphPad Software Inc.).

Results

There was a significant difference between cell viability curves with incubation time (df (1, 44), F = 146.70, $P = < 0.005$, Figure 4). The temperature range where time influenced activity was between 40 to 45°C. Below 37°C, both time points were insufficient to halt venom activity and above 48°C, 5 min incubation time was sufficient to destroy the venom activity. With 5 min incubation, the temperature required to neutralise venom from the 0.01% inhibitory concentration curve was 44.6°C (95% confidence limits of 44.5°C – 44.8°C). Incubating the venom for 20 min lowered the 0.01% inhibitory concentration temperature by approximately 2°C, requiring only 42.1°C (95% confidence limits of 41.9°C – 42.4°C) to inactivate the venom.

The average depth in which venom was released from the spine upon compression of the venom sacs was approximately 18 mm.

Discussion

Results demonstrate that exposure to heat significantly reduces the lethality of *S. horrida* venom through deactivation of venom components. More specifically, we demonstrated that exposure of *S. horrida* venom to temperatures above 39°C dramatically inhibits its cytotoxicity. This finding agrees with previous studies that suggest exposing venom to heat causes loss of its functionality and/or cytotoxicity.[20,25] Moreover, this research shows that decreases in immersion temperature lead to longer incubation periods to render the venom biologically ineffective.

The finding that the average depth in which venom is released from the glands is approximately 18 mm from the spine tip is relevant to the first-aid for stonefish envenomation. An increased incubation time will increase the probability of heat penetrating deeper into tissues and deactivating the venom that has been deposited without damaging profounder tissue. When comparing this study to the current generalised first-aid management protocol, it is plausible to say that hot water immersion (HWI) therapy could possibly resolve stonefish venom intoxications in 20 min instead of the proposed 90 min. Also, this can be achieved using a temperature at the lower range of the scale (i.e., 42°C). This should also minimize the chance of the victim getting a skin burn from hotter temperatures.[23]

On the other hand, by subjecting the patient to a shorter incubation time, a higher temperature will be required. In this case, patients can be at a greater risk of suffering first aid complications (i.e., scalding injuries) caused by exposure to damaging temperatures around 46°C.[26] Also, some patients cannot tolerate high temperatures and discomfort can result.[27] For these cases, further decreases in the HWI temperature could be helpful. Unfortunately, as this research only used two different incubation times, the time required to deactivate the venom at the 39°C threshold remains unknown. Moreover, further investigation using live animals is warranted to delineate both temperature and time thresholds *in vivo*.

Conclusions

An immersion time of 20 min at 42°C was sufficient to detoxify *S. horrida* venom in vitro. This could be recommended when clinicians select hot water immersion therapy to treat stonefish wounds. This procedure should maximise the successful deactivation of the toxin while minimizing the time in which the procedure is completed, diminishing the chances of the victim suffering secondary burns or discomfort.

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