An in-vitro examination of the effect of vinegar on discharged nematocysts of *Chironex fleckeri*

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Abstract

(Welfare P, Little M, Pereira P, Seymour J. An in-vitro examination of the effect of vinegar on discharged nematocysts of *Chironex fleckeri*. *Diving and Hyperbaric Medicine*. 2014 March;44(1):30-34.)

Objective: To determine the effect acetic acid (vinegar) has on discharged nematocysts in a simulated sting from *Chironex fleckeri*.

Method: This research was performed in 2 parts:

1 *C. fleckeri* tentacles placed on amniotic membrane were electrically stimulated, and venom washings collected before and after application of vinegar. Lyophilised venom washings were run through a fast-performance protein liquid chromatography column to confirm the venom profile, with a quantitative measure of venom from each washing calculated using UNICORN™ software.

2 The toxicity of the washings was determined by application to human cardiomyocytes, with percentage of cell detachment providing a measure of cell mortality, and hence toxicity.

Results: Part 1: There was a 69 +/- 32% ($F = 77$, $P < 0.001$) increase in venom discharge after vinegar was applied compared to post electrical stimulation.

Part 2: Venom collected after the administration of vinegar demonstrated the same toxicity as venom from electrically stimulated *C. fleckeri* tentacles and milked venom, causing cell mortality of 59 +/- 13% at 10 μg ml^{-1} compared to 57 +/- 10% and 65 +/- 7% respectively.

Conclusion: This in-vitro research suggests that vinegar promotes further discharge of venom from already discharged nematocysts. This raises concern that vinegar has the potential to do harm when used as first aid in *C. fleckeri* envenomation.

Key words

Jellyfish, envenomation, clinical toxicology, toxins, research, first aid

Introduction

Jellyfish envenoming is a major and increasing issue worldwide with numerous envenomations occurring each year, many of which require medical treatment.[1] *Chironex fleckeri* envenomations whilst relatively rare can be fatal, with more than 60 recorded deaths within Australian waters.[2] If stung by *C. fleckeri* (the large box jellyfish) in tropical Australia, the Australian Resuscitation Council (ARC) recommendation is to *"liberally douse/spray the sting area with vinegar (4–6% acetic acid) for 30 seconds."*[3] In the USA, vinegar is recommended as first aid for all jellyfish stings by the American Heart Association (AHA) and American Red Cross.[4]

The use of vinegar originated from laboratory studies on the tentacles from *C. fleckeri* in which vinegar was found to permanently inactivate all undischarged nematocysts, with later work attempting to isolate other compounds that may have a similar effect.[5,6] This reaction has been shown in several other cubozoans.[7–9] However, vinegar is also known to cause nematocyst discharge in other jellyfish species.[10–13] The inclusion of vinegar into resuscitation protocols is because of its beneficial action in inactivating undischarged cubozoan nematocysts, and through this process preventing further discharge and envenomation.[5] There is no dispute about vinegar's effectiveness in inactivating undischarged nematocysts of *C. fleckeri*; however, there are no published data to demonstrate vinegar has any benefit when applied to nematocysts which have already discharged. Discharged nematocysts are not innocuous; they are able to release further venom, for example, when pressure is applied.[14]

It is widely recognised that the application of vinegar does not reduce the symptoms of envenomation.[15] Vinegar has even been reported to worsen pain immediately after application and anecdotal reports from Cairns Base Hospital include increased analgesic needs in patient who have used vinegar on their sting site compared to those who had not been treated with vinegar.[16]

As a sting victim must have discharged nematocysts present, vinegar could be having a different effect on discharged nematocysts compared to its inactivation of undischarged nematocysts. This research was designed to ascertain how discharged nematocysts react to vinegar by quantitatively simulating human envenomation from *C. fleckeri*, with the application of 4% acetic acid to determine whether active venom is released after the application of vinegar and to determine its toxicity towards human cardiomyocytes.

Method

The study was approved by the Cairns Base Hospital Ethics Committee (approval number 287). This research was performed in two parts.

PART 1: VENOM PROFILES GENERATED FROM STIMULATED *C. FLECKERI* TENTACLES BEFORE AND AFTER THE APPLICATION OF VINEGAR

Venom Collection

To collect venom, we applied tentacles of *C. fleckeri* onto human amniotic membrane in an experimental procedure described previously.[14] In brief, human amniotic membrane was secured across one end of a sterile container from which the base had been removed to form an open-ended cylinder. Isotonic sterile saline (0.9% NaCl, 3.5 ml) was washed over the inside of the amniotic membrane five times to remove any extraneous proteins or foreign material, with the final washing kept for analysis (W1 – control).

Following this, 10 cm of tentacles from a freshly caught adult *C. fleckeri* were placed onto the outer surface of the amniotic membrane and partial discharge with adherence of the tentacles was observed. To maximise nematocysts discharge, we applied a 6-volt, 3-ampere direct current charge across the tentacle pieces for two seconds (Figure 1). Such electrical augmentation is currently used to collect venom from *C. fleckeri* for commercial anti-venom production.[17] Contraction and frosting of the tentacles were observed and taken as confirmation of successful nematocyst triggering and discharge.

Venom from the under surface of the membrane was then collected via washing in the following manner: the cylinder was inverted and 3.5 ml of 0.9% NaCl was placed into the cylinder, rinsing venom from the side of the membrane with the penetrating nematocyst shafts. The cylinder was agitated for 15 seconds, re-inverted and the washing collected (W2 – after voltage). To ensure clearance of discharged venom, the under surface was washed three times with 3.5 ml of 0.9% NaCl with the final washing kept for analysis (W3 – after wash).

The cylinder was re-inverted and 1 ml of 4% acetic acid (commercially available vinegar) was then applied to the adherent tentacles and left untouched for 30 seconds to simulate the current first-aid treatment guidelines recommended by the ARC.[3] Following this, the underside of the amniotic membrane was rewashed with 3.5 ml of 0.9% NaCl and the washing collected (W4 – after vinegar).

This entire experimental procedure was repeated twice, on new sections of the same amniotic membrane and with fresh tentacles from the same *C. fleckeri*, giving a total of three replicates. All washings were then lyophilised, weighed and stored at -80^{O}C.

Venom profiling

Known weights of each lyophilised sample were individually rehydrated to give solutions with protein concentrations of 0.27 mg ml^{-1} (to allow comparison with previously published data).[18] For some of the samples (W1 and W3), this required the majority of the sample to be rehydrated.

Figure 1

Electrical stimulation (6-volt, 3-amp DC) to discharge nematocysts of *C. fleckeri* tentacles placed on human amniotic membrane; toxin was obtained with washings of the underside of the membrane (see text for details)

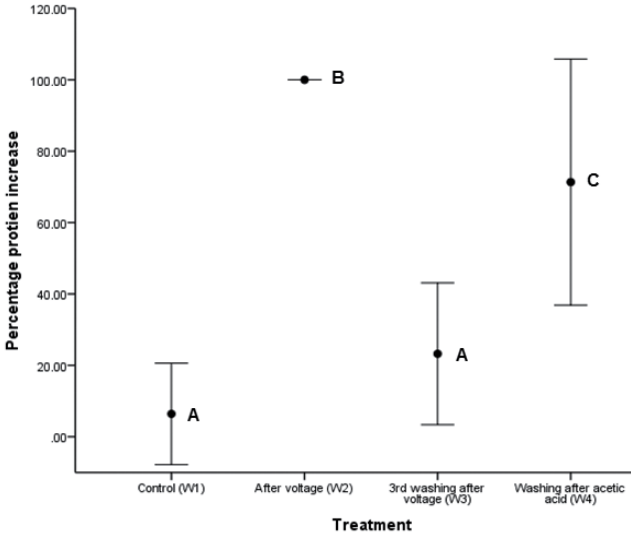
Between 200 and 500 µl of reconstituted venom was then passed through a 0.22 µm filter and individually run over an ÄKTA™ fast-performance protein liquid chromatography (FPLC) (Superdex ™ 10/200GL; Tricorn; 13 µm, 10 mm x 200 mm) at a flow rate of 0.3 ml min^{-1} and wavelength set at 280 nm, using degassed Dulbecco's phosphate buffered saline as a running buffer, and fractionated. This allows rapid purification of the proteins present within the venom, and the specific combination of proteins form a venom profile which can be compared to known data on *C. fleckeri* milked venom.[19]

Using UNICORN™ software, the area under the curve for the protein profile for each washing (listed above) was calculated and the total volume of venom expressed in each washing was back-calculated using the initial weight of lyophilised washing. These areas were then converted to percentages relative to the amount of protein collected after voltage was applied for each sample. Statistical analyses (one-way ANOVA) were performed on these percentages (transformed by arcsin square-root to normalise the distribution) to determine if the quantities of venom varied between the different washings. *Post hoc* analysis (using least-significant difference - LSD) was performed to determine which washings were statistically different to one another.

PART 2: APPLICATION OF VENOM TO HUMAN CARDIOMYOCYTES TO MEASURE TOXICITY

To allow the toxicity of the washings (W1, W2, W3 and W4) to be determined and compared to published results on *C. fleckeri* venom toxicity, the lyophilised washings were rehydrated. The lethality of these rehydrated washings was then tested using the Roche Applied Science and ACEA Biosciences Incorporated xCELLigence system. This system

Figure 2
Percentage increase in protein concentration found in washings (W1–W4); the protein (venom) found in the washing after electrical stimulation (W2) was significantly higher than any other washing. Vinegar applied after electrical discharge was associated with further protein expression (69 +/- 32% (F = 77, $P < 0.001$)); means with the same letter are not statistically different



quantifies cell survival by measuring cell attachment over time and has been used previously to show cell cytotoxicity of *C. fleckeri* venom.[18]

In brief, human cardiomyocytes were cultured in 75 cm^2 flasks. Once cell culture had reached 80% confluence, cells were lifted using a bovine trypsin solution. The number of cells in this solution was calculated using a haemocytometer and approximately 2000 cells with 150 µl of media were then seeded in each well of a 96 well E-Plate. Cells were incubated in the E-Plate for 24 hours at 37^{O}C and 5% carbon dioxide to ensure all cells were properly attached to the base of the wells before commencing experimentation. Reconstituted washings were applied to individual wells and cell survival was determined as described previously.[18,19] (N.B. Washings were tested at a concentration of 10 µg ml^{-1} for direct comparison with a previous study.[18] However, the control (W1) and W3 had insufficient protein to achieve this and instead were tested at a concentration of 1 µg ml^{-1}.

Results

PART 1

The venom profile obtained from the FPLC was similar to that reported previously for *C. fleckeri*.[19] Venom was detected post electrical stimulation and after application of vinegar. This study demonstrated that the application of vinegar to a *C. fleckeri* tentacle that had been electrically discharged was associated with further protein (venom) expression of 69 +/- 32% more protein (venom) (Figure 2).

Table 1
Percentage toxicity (95% confidence limits) of different dilutions of washings from *Chironex fleckeri* envenomed amniotic membrane against human cardiomyocytes compared to extracted (milked) whole *C. fleckeri* venom (data taken from reference 18)

	% cell death at 10 min (10 mg ml^{-1})	% cell death at 10 min (1 mg ml^{-1})
Control washing (W1)	n/a	0
1st washing after voltage (W2)	57 (47, 67)	21 (12, 30)
3rd washing after voltage (W3)	n/a	0
Washing after vinegar (W4)	59 (46, 72)	16 (7, 25)
Milked venom	65 (58, 72)	19 (11, 27)

There was a significant difference between the protein concentrations of the different treatments ($F = 77.12_{3x82}$, $P < 0.001$). The protein (venom) found in the washings after voltage was applied (W2) was significantly higher than all other treatments (LSD, $P < 0.001$). Similarly, the percentage increase in protein (venom) found in the washings after vinegar was applied (W4) was significantly higher than controls (W1, W3) (LSD, $P < 0.001$) but not as high as in washings after electrical stimulation (W2) (LSD, $P = 0.001$) .

PART 2

Washings post electrical stimulation (W2), and post application of vinegar (W4) were toxic to human cardiomyocytes. These washings had activity similar to previously published studies using whole extracted *C. fleckeri* venom (Table 1).[18] This activity decreased to the levels of the control (W1) in subsequent washings (W3) and recrudesced in washings collected after the application of vinegar (W4).

Discussion

Previous research has shown that application of weak (3–10%) acetic acid for 30 seconds to *C. fleckeri* tentacles does not trigger discharge of nematocysts, and that nematocyst discharge from undischarged nematocysts is irreversibly inhibited.[5] It is unknown why this occurs, but it is postulated to be due to the terminal carboxyl group.[5] This action is not refuted by this study. Instead, we have confirmed earlier findings that triggered (or discharged) nematocysts are incompletely discharged of venom.[14]

More importantly, the application of vinegar was associated with further discharge of venom. We are unsure as to why this occurs, but given there is evidence that vinegar completely inactivates undischarged nematocysts we can only postulate that this additional venom has been expressed from discharged nematocysts, perhaps through a chemically mediated process of nematocyst wall

contraction. Furthermore, this venom exhibited the same cardiomyotoxic activity as the initial venom discharged. This finding may explain why the application of vinegar gives no symptom relief and may in certain cases (e.g., when a large proportion of discharged nematocysts is present compared to undischarged) actually exacerbate pain. However other causes, e.g., the application of a mild acid to already damaged skin, could also increase pain experienced by the patient.

This raises concern that vinegar may be harmful when applied as first aid to a sting victim who has both discharged and undischarged nematocysts present on their skin. It is unknown what the proportion of discharged to undischarged nematocysts is on a patient with envenomation, and ideally undischarged nematocysts should be inactivated with vinegar. However, if vinegar causes further discharge in already discharged nematocysts in vivo, vinegar may or may not have an overall benefit.

Therefore, it may be time to reconsider first-aid options for tropical Australian jellyfish stings. Heat (hot water) can lessen the pain experienced from bluebottle *(Physalia*) stings, and other types of box jellyfish (*Carybeda alata*).[20–23] In animal models, hot water has been shown to stop the lethal effects of *C. fleckeri* venom when heated above 43°C.[24] However, the ability to obtain hot water in a timely fashion, and the high temperatures required limit its feasibility as a first-aid measure. Topical lidocaine has been shown to be an effective analgesic in stings from the box jellyfish *Chiropsalmus quadrumanus* (sea wasp).[10] Lidocaine is also proven to inhibit nematocyst discharge in *Chrysaora quinquecirrha* (sea nettle) and *Physalia physalis* (Portuguese man-of-war).[10] To date, there are no studies into the use of lidocaine on *C. fleckeri*.

Conclusion

This in-vitro research demonstrates that vinegar promotes further discharge of venom (approximately a further 69% of venom load released) from already electrically discharged nematocysts of *C. fleckeri*. This in turn raises concern that vinegar may have the potential to do harm by exacerbating envenomation from *C. fleckeri*. Further investigations are required to elucidate the mechanism(s) of this secondary release of toxin and to identify first aid measures which will reduce both pain and the risk of cardiac arrest.

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Conflicts of interest: Nil.

Submitted: 13 November 2013
Accepted: 04 January 2014

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