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Rapid and permanent cytotoxic effects of venom from *Chiropsella bronzie* and *Malo maxima* on human skeletal and cardiac muscle cells

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ABSTRACT

Jellyfish envenomation is a global public health risk; Cubozoans (box jellyfish) are a prevalent jellyfish class with some species causing potent and potentially fatal envenomation in tropical Australian waters. Previous studies have explored the mechanism of action of venom from the lethal Cubozoan *Chironex fleckeri* and from C*arukia barnesi* (which causes "Irukandji syndrome"), but mechanistic knowledge to develop effective treatment is still limited. This study performed an *in-vitro* cytotoxic examination of the venoms of *Chiropsella bronzie* and *Malo maxima*, two understudied species that are closely related to *Chironex fleckeri* and *Carukia barnesi* respectively. Venom was applied to human skeletal muscle cells and human cardiomyocytes while monitoring with the xCELLigence system. *Chiropsella bronzie* caused rapid cytotoxicity at concentrations as low as 58.8 μg/mL. *Malo maxima* venom caused a notable increase in cell index, a measure of cell viability, followed by cytotoxicity after 24-h venom exposure at ≥11.2 μg/mL on skeletal muscle cells. In contrast, the cardiomyocytes mostly showed significant increased cell index at the higher *M. maxima* concentrations tested. These findings show that these venoms can exert cytotoxic effects and *Malo maxima* venom mainly caused a sustained increase in cell index across both human cell lines, suggesting a different mode of action to *Chiropsella bronzie*. As these venoms show different real-world envenomation symptoms, the different cellular toxicity profiles provide a first step towards developing improved understanding of mechanistic pathways and novel envenomation treatment.

1. Introduction

Box jellyfish, class Cubozoa, inhabit the coastal and ocean waters predominantly between the tropic of Cancer and Capricorn (Gershwin et al., 2010; Tibballs, 2006). In Australia, most human envenomation occurs between October to May with occasional exceptions (Tibballs, 2006; Barnes, 1967; Burnett et al., 1998; Courtney and Seymour, 2013; Currie, 2003). The two orders within Cubozoa, Chirodropida and Carybdeida, are well known for their potent, potentially fatal, venoms which cause a diverse range of symptoms in human envenomation as shown in Fig. 1 [6-9]. The most well-documented human envenomations from Cubozoa are caused by *Chironex fleckeri*, the large box jellyfish, and *Carukia barnesi*, the "Irukandji" box jellyfish (Tibballs, 2006, 2018; Tibballs et al., 2012; Currie and Jacups, 2005). These box jellyfish may cause mild to severe symptoms in humans which may result in hospitalization and fatality (Tibballs, 2006; Currie and Jacups, 2005). This study aimed to monitor cellular response when challenged with less well studied Cubozoan venoms, *Chiropsella bronzie* and *Malo maxima* (Fig. 1).

Chirodropid envenomation may result in severe peripheral pain where the nematocysts (stinging cells) contact the skin during envenomation, and cardiotoxicity which may be fatal. More than 77 documented fatalities, including in recent years, have occurred due to *C. fleckeri* envenomation in the Northern Territory and Queensland (Currie and Jacups, 2005; Currie, 1994). Envenomation by the closely related species, Chiropsella *bronzie* (*C. bronzie*) has been less studied than *C. fleckeri* venom (Fig. 1). Studies on the venom of *C. bronzie* in animals describe haemolytic and dermatonecrotic symptoms after venom exposure (Koyama et al., 2003; Ramasamy et al., 2005a). The venom caused a bi-phasic pressor response and cardiovascular collapse in anaesthetized rats (Ramasamy et al., 2005a), and induced apoptosis in rat malignant glioma cell lines (Sun et al., 2002). However human envenomation appears to be less severe with only mild pain, redness and itching reported in human envenomation (Tibballs, 2006). *Chiropsella bronzie* was incorrectly referred to as *Chiropsalmus quadrigatus*, in Australia until 2006 (Gershwin, 2006).

In contrast, some species cause mild pain and welts while other

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species of Carybdeida causes Irukandji syndrome upon envenomation; a condition with symptoms including prolonged systemic pain which may include muscle pain and cramps, vasoconstriction, hypertension, headaches, sweating, abdominal pain, vomiting and systemic inflammation (Tibballs et al., 2012; Flecker, 1952; Barnes, 1964; Ryan et al., 2021). Irukandji envenoming routinely require hospitalization even though they are generally not fatal, however, two fatalities from Irukandji envenoming have occurred due to intracerebral hemorrhage (Pereira et al., 2010; Fenner and Hadok, 2002). One of the fatalities was attributed to *C. barnesi* (Huynh et al., 2003). The related species *M. maxima*, family Carukiidae, has been linked to Irukandji syndrome (Tibballs et al., 2012; Gershwin, 2007). A pharmacological study of *M. maxima* venom reported parasympathetic nervous system activation resulting in left atrial bradycardia and increased nerve calcitonin gene-related peptide release, which increases nociception (Li et al., 2011). The authors concluded that these findings were consistent with the previously proposed excess catecholamine release mechanism for Irukandji syndrome. Therefore, *Malo maxima* may be responsible for the Irukandji syndrome cases reported in Western Australia (Li et al., 2011).

Antivenom is available for *C. fleckeri* envenomation. However, a clinical case study has reported that only 5% of jellyfish envenomed patients received antivenom and unfortunately there are four documented deaths despite antivenom administration (Currie, 2003). Thus, the antivenom modality has been called into question by the scientific and medical community (Currie, 2003; Currie and Jacups, 2005; Ramasamy et al., 2003; Winter et al., 2009; Konstantakopoulos et al., 2009). It has also been proposed that antivenom may be too slow to be therapeutic for the rapid-acting, potentially fatal venom; a cell assay study reported a lengthy delay before the intended neutralizing effect of the antivenom occurred (approximately 70 min) (Piontek et al., 2020; Winter et al., 2009; Andreosso et al., 2014). *Chironex fleckeri* antivenom was found clinically ineffective at treating Irukandji syndrome (Fenner et al., 1986), and it seems unlikely any antivenom for the order Carbdeida will be produced due to challenges such as which species was responsible for envenomation (Ramasamy et al., 2005b). Thus, treatment for severe box jellyfish envenomation heavily relies on hospital supportive care and opioids for pain management (Huynh et al., 2003). Improved treatment for all box jellyfish envenomation is urgently needed.

Due to the distinct human envenomation symptoms reported between the orders Chirodropida and Carbdeida, the present study aims to identify cytotoxic effects of the understudied *C. bronzie* and *M. maxima* venoms. Cubozoan venom profiles and potency are affected by the time of year collected and geographical location (Winter et al., 2010). Other animals, such as rattlesnakes, have also shown venom modulation dependent on environmental factors (Zancolli et al., 1898). Thus, this study will only use a single collection pool to comprise the venom sample for each species. This study monitors the effect of the venoms on Human Cardiomyoctyes (HCM) and Human Skeletal Muscle Cells (HSkMC) using a real-time cell monitoring assay, the xCELLigence system. The xCELLigence assay has been employed successfully for *in vitro* studies of related jellyfish venom effects on human cell lines (Ramasamy et al., 2003; Pereira and Seymour, 2013; Chaousis et al., 2014; Saggiomo and Seymour, 2012). This study will show a similar response to previously published cell monitoring studies examining the effects of *C. fleckeri* and *C. barnesi* venoms (Pereira and Seymour, 2013; Chaousis et al., 2014; Saggiomo and Seymour, 2012).

2. Methods

2.1. Venom collection & extraction

Live *C. bronzie* were sampled from Wonga Beach (16.3181° S, 145.4091° E), north of Port Douglas, Queensland. Live *M. maxima* were sampled from waters at the Port Douglas marina (16.2913°S, 145.2732°E). For this study, venom was pooled from several animals to create a consistent bulk venom supply. The nematocysts were collected and extracted as per Bloom et al. (1998). In brief, tentacles were stored in refrigerated seawater (4 °C) for 5 days, resulting in spontaneous detachment of the nematocysts into the seawater. The tentacles were removed from the seawater and the solution was strained with a fine kitchen sieve. The solution was washed with fresh seawater and was allowed to settle for 3 h. The resulting sediment of nematocysts was lyophilized and stored at −20 °C until needed. Venom was extracted from the lyophilized nematocysts as described by Carette et al. (Carrette and Seymour, 2004). Lyophilized nematocysts, 0.005g, were rehydrated in 3 mL microcentrifuge tubes with 1.5 mL filtered deionized water (MQ water, Milli-Q-Ionized Filter, Merck, USA), and approximately 1.3 mL glass beads (0.5 mm Biospec Products, USA). To mechanically disrupt nematocysts the sample was shaken in a Mini-Bead-Beater (Biospec Products, USA) at 725g ten times for 2 min, with a resting period on ice for 5–7 min between shaking. As per Carette et al., the tubes were centrifuged at 3000 rpm for 1 min to pellet the heavier capsular debris. Venom was decanted from the tubes, leaving behind heavier materials. The supernatant was filtered through a 0.45 μm PVDF filter (Merck Millipore, USA) to remove any remaining debris. The sample was lyophilized and stored at −80 °C until further use.

2.2. Cell culture & xCELLigence plate seeding

Human skeletal muscle cells (HSkMC Cat. #3500) and human cardiomyocytes (HCM Cat. #6101) were cultured according to ScienCellTM recommendations in the respective ScienCell media (ScienCellTM) with 10% fetal bovine serum, Cardiac Myocyte Growth Supplement 100x, and penicillin and streptomycin antibiotics in 25 cm^2 flasks at 37 °C per the manufacturer's instructions. Cell index, a measure of cell adhesion to the bottom of the wells, was monitored using the xCELLigence system. As cells proliferate, the xCELLigence monitors cell attachment to plates

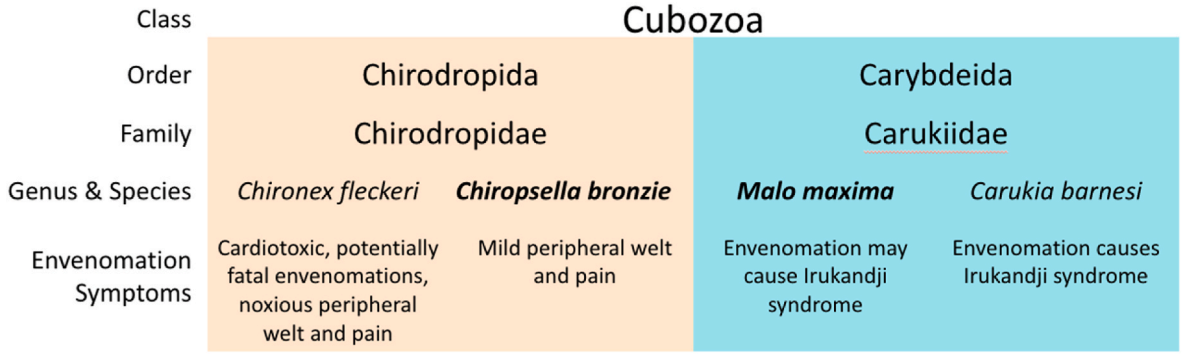

Fig. 1. Chart of scientific classification and human envenomation symptoms reported for the box jellyfish of interest (6–9). Venom from *Chiropsella bronzie* and *Malo maxima* was analyzed in this study.

by measuring resistance in real-time. A 96-well gold-plated e-well plate (ACEA Biosciences, Santa Clara, California, USA) was used in all experiments. The plate was seeded with 3000 cells per well, in 150 µL media, 24 h before venom application to allow cell adherence. Lyophilized venom for both species was rehydrated with MQ water, centrifuged for 10 min at 855×*g* at 4 °C and was filtered with a 0.22 µm PVDF Millipore Filter. Stock concentrations were limited based on the sample collected at the beach. The *C. bronzie* venom concentrations tested ranged from 2 µg/mL to 176.4 µg/mL, while *M. maxima* venom concentrations ranged 2 µg/mL to 65.9 µg/mL. Venom concentrations were measured using a Nanodrop One (ThermoFisher Scientific, USA) at 280 nm absorbance. All concentrations were tested in triplicate on both cell lines. Control wells, containing cells treated with only the vehicle Dulbecco's Phosphate Buffered Saline (DPBS). To closely monitor initial venom effects, 50 xCELLigence reads were taken for the first 15 s, followed by 50 reads every 2 min for 15 min. Subsequently, 100 reads were taken every 15 min until 24 h and 100 reads every hour to monitor long-term responses after 24 h. Data were analyzed using the xCELLigence Real Time Cell Analysis (RTCA) software, V2 (ACEA Biosciences, Inc., 2013). To test the effects of venom concentration on cell survival, dose response curves were produced by monitoring replicates of every concentration.

2.3. The xCELLigence assay

The xCELLigence, RTCA, Single Plate System (ACEA Biosciences, Inc., Santa Clara, California) provided real-time bio-impedance measurements for the cell index, a measure of cell viability, of both human skeletal muscle cells and human cardiomyocytes dosed with venom and control, DPBS. Data outputs from xCELLigence were recorded with the RTCA software. Data are shown as mean normalized cell index, at the point of treatment additions, and standard error of the mean (S.E.M.) with cell index = 1 representing the cell index measurement just prior to toxin addition.

2.4. Analysis

2.4.1. Changes in cell index over time at four venom concentrations

Averaged, normalized xCELLigence cell index data (Fig. 2) were created to compared venom concentrations (*C. bronzie* 58.8 and 178.4 µg/mL, *M. maxima* 44.9 and 65.9 µg/mL) to control, DPBS, with a two-way ANOVA (time x concentration) and Holm-Sidak's multiple comparison: * = $p \leq 0.05$; ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$.

2.4.2. Analysis of covariance and all possible interactions on cell viability at 24 h

The relationship between venom concentration and xCELLigence readings appeared linear for each of the four combinations of jellyfish species and cell type, therefore an analysis of covariance was used to model those relationships and to compare the slopes and intercepts of each of the four regressions of cell index against venom concentration. An initial analysis therefore used venom concentration, species, and cell type as explanatory variables, and included all possible interactions between them. As the three-way interaction proved to be highly significant, we then analyzed data from each jellyfish species separately.

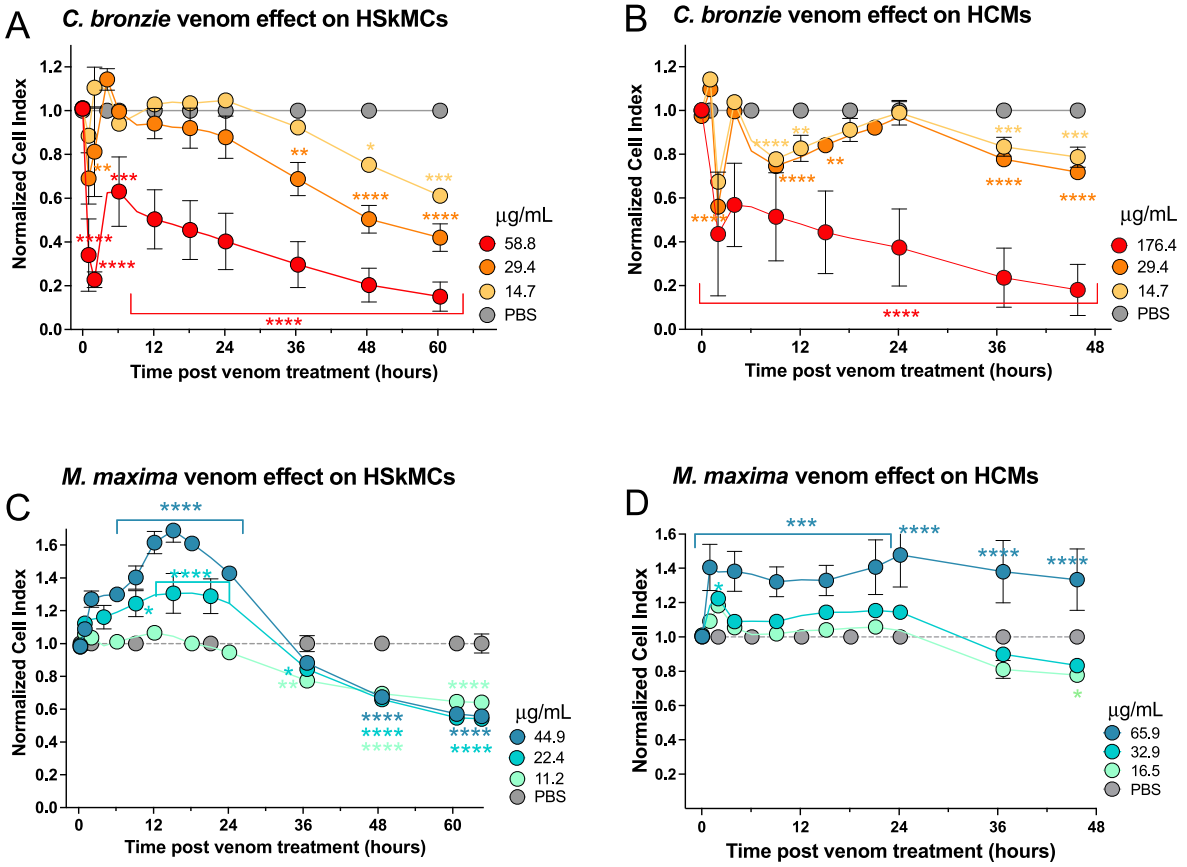

Fig. 2. Timed cell viability response to venom application. (**A.**) *Chiropsella bronzie* venom effect on Human Skeletal Muscle Cells (HSkMCs) and Human Cardiomyocytes (HCMs) (**B.**). *Malo maxima* venom application on HSkMCs (**C.**) and HCMs (**D.**). Data plotted as normalized Cell Index (nCI), a measure of cell viability, relative to DPBS control vs. time. Statistical comparison made with a two-way ANOVA, with Holm-Sidak's multiple comparison against DPBS control: * = $p \leq 0.05$; ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$. Error bars on each data point indicate standard error of the mean with n = 3 for all data points. Some error bars are not visible as they are smaller than the symbol, some points have been nudged (± 0.5 h) to aid data visualization.

All analyses used R (R Core Team, 2017).

2.4.3. Cell index and dose response curve after 24 h venom exposure

Some of the four combinations of jellyfish venom and cell type exhibited a non-linear correlation between venom concentration and xCELLigence readings at the 24-h mark. Therefore, an analysis of covariance comparing the regression curves was employed to model these relationships and compare cell index values at each venom concentration for each cell type and species. An initial analysis therefore used venom concentration, species, and cell type as explanatory variables, and included all possible interactions between them. As the three-way interaction proved to be highly significant, we then analyzed data from each jellyfish species separately. All analyses used R statistical software (R Core Team, 2017).

2.4.4. Changes in dose response curve (DRC) and IC_{50} over time

The normalized cell index (nCI), a measure of cell viability was plotted relative to the control, DPBS vehicle diluent, and graphed in Prism (Graphpad, v 8) to produce dose response curves (DRCs). DRCs were fitted with a variable hillslope, bottom constraint set to approximately double the maximum venom response and the top constraint was set to zero. The IC_{50} statistical comparison was made with a sum of squares F test and a Bonferroni correction, similar to previous *C. fleckeri* DRCs reported in previous studies (Chaousis et al., 2014). The IC_{50} was only able to be generated for *C. bronzie*; as the majority of data for *M. maxima* venom showed an increasing cell index response.

2.5. Size exclusion chromatography of the cubozoan venoms

Lyophilized venom was rehydrated with milliQ water, and centrifuged for 10 min at 855×*g* and 4 °C. The venom was then filtered with a hydrophobic PVDF membrane 0.22 μm Millipore Filter. The *C. bronzie* venom concentration was 159.0 μg/mL and *M. maxima* venom concentration was 44.9 μg/mL. Both venoms were then separated by fast protein liquid chromatography using a size exclusion column (SuperDex 200 Increase 10 × 300 mm) on an AKTA system (GE Healthcare). Samples (159.0 μg of *C. bronzie* venom and 44.9 μg of *M. maxima* venom) were run in 500 μl PBS buffer at 0.5 mL/min flow and monitored with an absorbance wavelength of 280 nm. Fraction (0.5 mL) were collected in 96-well deep-well plates (Corning, 1 mL). Venom profiles were processed using the UNICORN 5.2 software version 1.3 (2008, GE Healthcare).

3. Results

3.1. Cell response to cubozoan venom application over time

The venom of *C. bronzie* and *M. maxima* was tested for cytotoxicity with two human cell lines, HCM and HSkMCS, using cell viability real-time assay, the xCELLigence system. Plots of averaged, normalized xCELLigence cell index data show *C. bronzie* and *M. maxima* venom effects on HCMs and HSkMCs compared to PBS controls (Fig. 2). *Chiropsella bronzie* caused immediate loss of cell index, reflecting cytotoxicity (visual inspection, not shown) at 58.8 μg/mL on HSkMCs and 176.4 μg/mL on HCMs (Fig. 2 A and B). HSkMC index declined steadily throughout the experiment. In comparison, lower concentrations of *C. bronzie* venom (14.7 and 29.4 μg/mL) initially caused a cell index decline, followed by recovery to control at approximately 24 h. HCM index decline after 36 h was less substantial compared to HSkMCs (Fig. 2C). Notably, *M. maxima* venom caused a rapid substantial cell index increase at the highest tested concentrations, 44.9 and 65 μg/mL in HSkMCs and HCMs respectively (Fig. 2C and D). In addition, in HSkMCs, *M. maxima* venom subsequently caused loss of cell index and cytotoxicity (visual inspection, not shown), after 24 h at concentrations of ≥11.2 μg/mL. The *M. maxima* venom induced a continuous cell index increase of HCMs until the end of the experiment at 42 h, with an overall change of 0.8 cell index units at 65.9 μg/mL (Fig. 2D).

3.2. Initial effects of concentration, cell type, jellyfish species and their interactions on cell viability after 24 h

The initial regression analysis examined the effects of concentration, cell type, jellyfish species and all possible two- and three-way interactions on cell viability. An analysis of variance of the regression found a significant three-way interaction between concentration, cell type, and jellyfish species, confirming that the effect of cell type on the regression differed between the two jellyfish species (Fig. 3). Each species was, therefore, analyzed separately, and used a quadratic term to test for curvature in each case. The coefficients for both the linear and quadratic term were allowed to vary with cell type. This model was then simplified to achieve the minimum adequate model in each case.

For *C. bronzie* venom (Fig. 3A), there were no significant interactions but all main effects achieved statistical significance (for venom concentration $F_{1,64} = 426.2$, $p \ll 0.00001$; for venom concentration (Tibballs, 2006) $F_{1,64} = 41.91$, $p < 0.00001$; for cell type $F_{1,64} = 7.72$, $p = 0.007$). The model accounts for 88% of the variation in cell viability.

For the two cell types there is a common decelerating decline in viability as *C. bronzie* venom concentration increases.

Response to *M. maxima* venom exposure was different from *C. bronzie* venom (Fig. 3)., with cell index increasing as venom concentration increased. There was no evidence of curvature in either regression line for *M. maxima* venom when the interaction term was allowed for (Fig. 3B), possibly because the higher venom concentrations were not tested in this species due to available sample, but both the slope and intercept of the regression differed between cell types (for venom concentration $F_{1,49} = 114.28$, $p \ll 0.00001$; for cell type $F_{1,49} = 62.97$ $p < 0.00001$; for the interaction term $F_{1,49} = 29.32$, $p < 0.0001$). The model accounts for 81% of the variation in cell viability.

Diagnostic plots indicated that there were more outlying values than was consistent with the assumption of normally-distributed residuals, and that the outliers were predominantly at *M. maxima* venom between concentrations 35 μg/mL and 8.75 μg/mL. Omitting data with venom concentrations in this range normalized the residuals but did not alter the factors influencing cell viability.

3.3. Changes in C. bronzie venom over time dose response curve (DRC)

The cytotoxic effect of *C. bronzie* venom on cell lines increased with concentration when applied to both HCM and HSkMC cell lines (Fig. 4), but the loss of cell index rate varied between the cell lines (Fig. 4. A&B). At the 10-min point, venom concentrations from 14.7 μg/mL to 160 μg/mL showed toxicity on HCMs, while no toxicity was seen in HSkMCs at this early timepoint. This early toxicity, examined because the venoms are known to have rapid action, was reflected in the significantly lower 10-min IC_{50} for HCMs ($p < 0.0001$) (Fig. 4C). At 1-h this difference was reversed with a marked drop in IC_{50} for the HSkMCs, however this difference between the two cell lines was not significant at 1 h and the remaining times. Despite these early differences, from the 24-h time point the dose response curves for the cell lines was not significantly different. Finally, HSkMCs lost cell index at lower concentrations of *C. bronzie* venom, with 58.8 μg/mL causing 40–80% cell index loss relative to control (Fig. 4B), compared to the higher 176.4 μg/mL required to decrease the HCM cell index by similar percentages (Fig. 4A).

3.4. Changes in M. maxima dose response curve (DRC) over time

Dose response curves for *M. maxima* venom on HCMs and HSkMCs were determined for comparison between the cell lines (Fig. 5). The venom caused a substantial and prolonged increase in HCM cell index at all times after 1 h. Of particular note is the approximate 20–50% rise from 6 to 45 h at the highest concentration of 65.9 μg/mL. While the cell

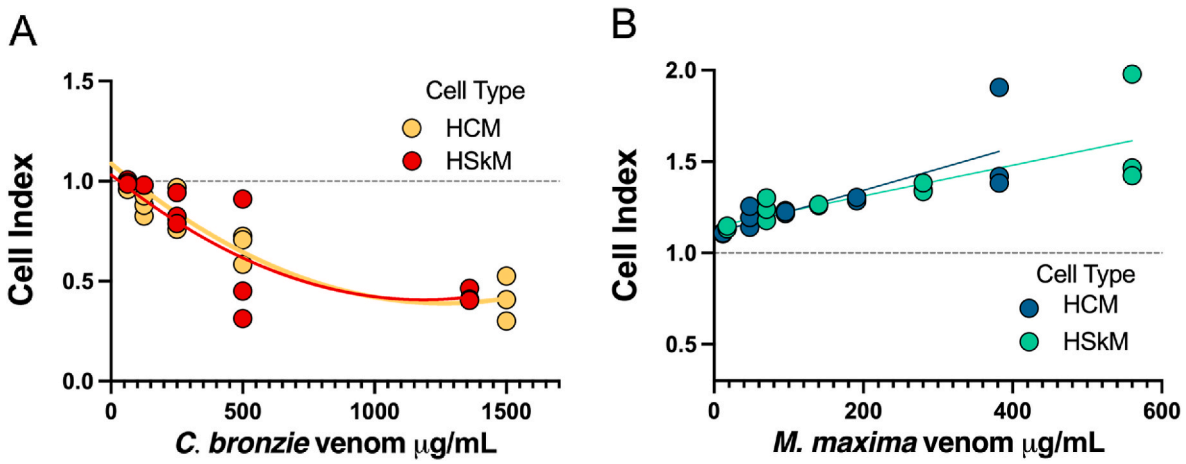

Fig. 3. Initial 24-h effects of venom concentrations on cell index, a measure of cell viability. (**A**) *C. bronzie* venom quadratic regression vs. (**B**) *M. maxima* venom linear regression: graphs were compared for both Human Cardiomyocytes (HCMs) and Human Skeletal Muscle Cells (HSkMCs) with an analysis of covariance. Graphs show each replicate, plotting the effect of venom concentration on cell viability for each combination of both cell types and each jellyfish species venom at 24 h. The two cell type regressions differed significantly for *M. maxima* but not for *C. bronzie*.

index rise was similar from 6 to 24 h for HSkMC, at time points after 24 h results were indicative of toxicity. Cell index readings dropped below controls at concentrations: 44.9, 22.4 and 11.2 μg/mL, in contrast to the HCMs. HSKMC retained 35–50% permanent loss of cell index compared to controls through the final timepoints (see Fig. 2C). Limited toxicity at final timepoints did not allow for IC_{50} calculations.

3.5. Size exclusion separation: distinct protein profiles for individual cubozoans

Each venom sample was separated independently using size exclusion liquid chromatography (Fig. 6). The separations showed similar protein peak profiles (Fig. 6). Peak No. 6, near 21.8 mL, is the salt peak and is indicative of zero kilodalton size of conventionally separated proteins (Housley et al., 2020). The salt peak is at 21.8 mL based on size standards (Precision Plus Protein™ Dual Color, BioRad) and our experience in Housley et al. and Lennox-Bulow et al. (Housley et al., 2020; Lennox-Bulow et al., 2022). After 21.8 mL, the portions of absorption are distinct between the two species with peaks 6–10 substantially differing in relative heights. The relative peak heights of peaks 6–9 were 4.5–12-fold higher in *M. maxima* venom, while peak 10 was only present in *C. bronzie* venom.

4. Discussion

This study is the first to present an analysis of *C. bronzie* and *M. maxima* venom effects on human cells. *C. bronzie* venom was more toxic in in HSkMC compared to HCM, causing permanent reduction in cell index. HCMs recovered at lower concentrations of *C. bronzie* venom but higher concentrations also caused a permanent reduction in cell index, a measure of cytotoxicity (Chaousis et al., 2014; Saggiomo and Seymour, 2012; Xing et al., 2006; Neale et al., 2018). However, this study shows that *M. maxima* venom caused an initial increase in cell index in both cell lines. This increase was only sustained in HCMs, while cytotoxicity was also noted after long exposure in HSkMCs. This study is the first to show *M. maxima* venom caused cytotoxicity in HSkMCs. In both cell lines, *C. bronzie* was cytotoxic in the first hour, as had been previously reported for *C. fleckeri* venom (Chaousis et al., 2014; Saggiomo and Seymour, 2012), however effects after 1-h were consistent for these species and both cell lines (Saggiomo and Seymour, 2012). However, *C. fleckeri* venom caused 100% cell death and was far more toxic to HCM compared to HSkMC, while our data show *C. bronzie* had similar toxicity to both muscle cell types. Venom components between the species were compared with size exclusion liquid chromatography and showed the protein peaks were similar (1–5), while the smaller compound peaks (6–10) showed substantial difference in relative abundance between the two species.

C. bronzie and *M. maxima* venom chromatograms showed similar peaks (1–5) to previously reported *C. fleckeri* venom (Chaousis et al., 2014); all three venom samples were separated by size exclusion liquid chromatography. However, both venoms from this study also showed an abundance of smaller compounds peaks (6–10) than were reported for *C. fleckeri* venom. In this study, both variations in the components of venom for each species and the cell model tested may contribute to the various responses observed; each cell line has unique receptor expression etc. Previous research in *C. fleckeri*, has identified two pore-forming toxins which are likely responsible for the pain, welts and scarring in severe human envenomation (Brinkman and Burnell, 2007). Despite the discovery of pore-forming proteins, effective treatment has yet to be developed and *C. fleckeri* envenomation causes approximately one fatality annually in Australia (Currie, 1994; Keesing et al., 2016). Future research should endeavor to identify the causative venom components in the Cubozoan species using analytical chemistry techniques, as has begun for *C. fleckeri* venom (Brinkman and Burnell, 2007). Identification of venom components may contribute to the development of novel treatment for human envenomation.

The impact of venom on cell proliferation was evaluated using the xCELLigence system to monitor adherent cell attachment to plates by measuring impedance in real-time reported as cell index. The loss of cell index indicates several cellular responses that have an effect on the electrical current flow between the direct contact of the adherent cell and the sensing micro-electrodes (Xing et al., 2006). Venom-induced reductions in cell index have been correlated to toxicity in previous research (Chaousis et al., 2014; Saggiomo and Seymour, 2012; Xing et al., 2006; Neale et al., 2018).

Chiropsella bronzie caused up to 75% cytotoxicity at 58.8 μg/mL. However, *C. bronzie* venom was not as cytotoxic when compared to the closely related *C. fleckeri* venom which, in a previous study, was shown to cause 50% cell index loss at approximately 10 μg/mL (Saggiomo and Seymour, 2012). In the current study, the regression comparison of cell line response to venom application showed that the *C. bronzie* IC_{50}, in HCM showed a significantly lower (more toxic) IC_{50} at 10 min, 1 h and 6 h compared to HSkMCs ($p < 0.0001$). IC_{50} was examined as early as 10 min because of the known rapid action of Cubozoa venoms. In rodent models, other studies have shown a similar potent cardiac effect with both *C. fleckeri* and *C. bronzie* venoms producing irreversible contraction

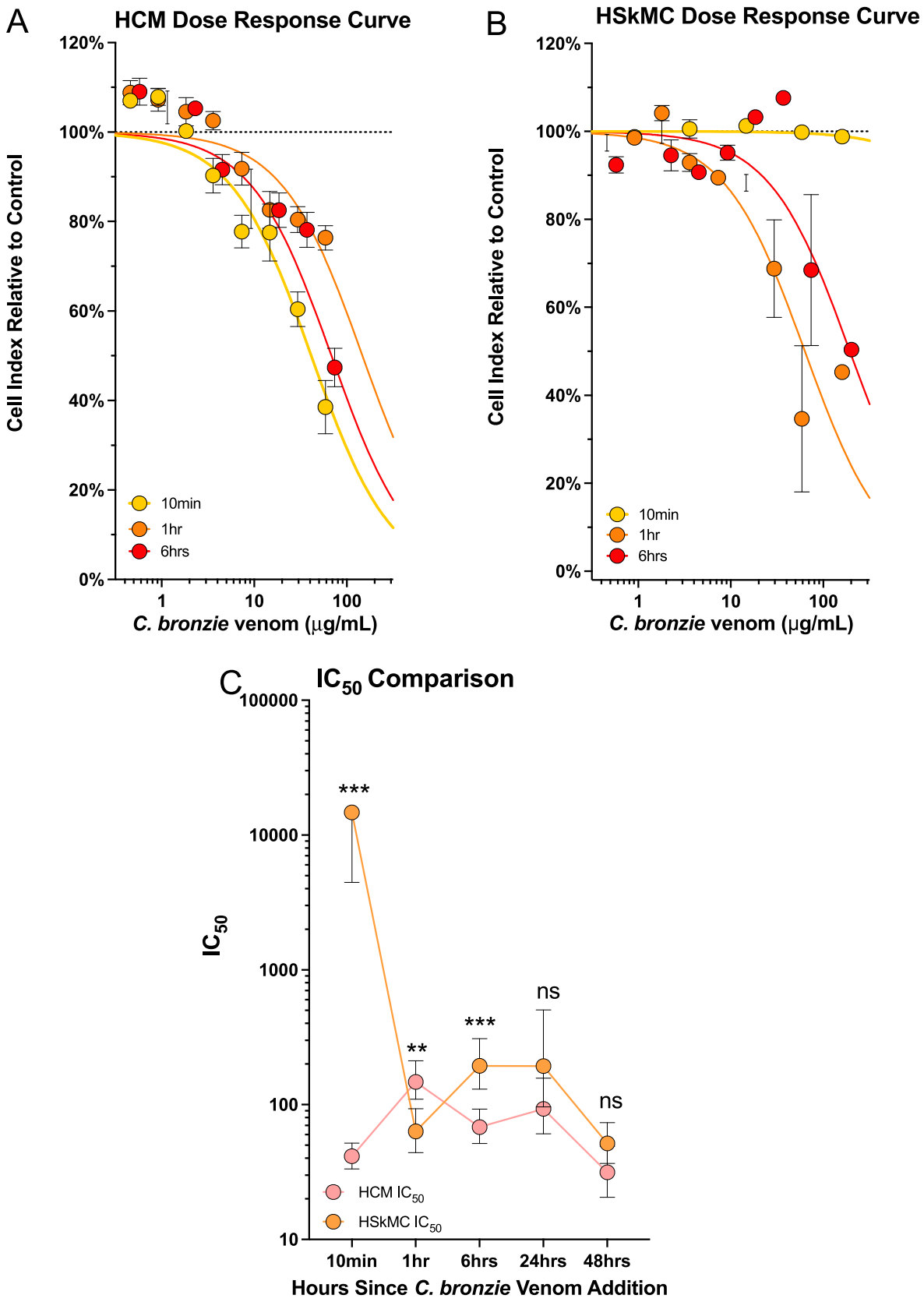

Fig. 4. Comparison of cell line dose responses to *C. bronzie* venom. Dose Response Curve of *C. bronzie* venom on Human Cardiomyocytes (HCM) shown on x-axis, antilog (**A.**) and Human Skeletal Muscle Cells (HSkMC) (**B.**) relative to control generated for the time course the cells had been exposed to venom, the dashed line at 100% shown to aid data visualization. Panels A and B: Error bars on each data point indicate standard deviation around the mean, (n = 3). **C.** Comparison of the IC_{50}s (median Inhibitory Concentration) of *C. bronzie* venom over time (10 min–48 h) HCMs and HSkMCs calculated from Panels A-C with 95% confidence interval error bars. The IC_{50} for both cell lines were compared at each data point using a sum of squares F-test and manual Bonferroni correction: *** = $p \leq 0.001$, ** = $p \leq 0.01$, NS = not significant. The top error bar at 10 min for HSkMCs was not calculable, other error bars not visible as they are smaller than the symbol.

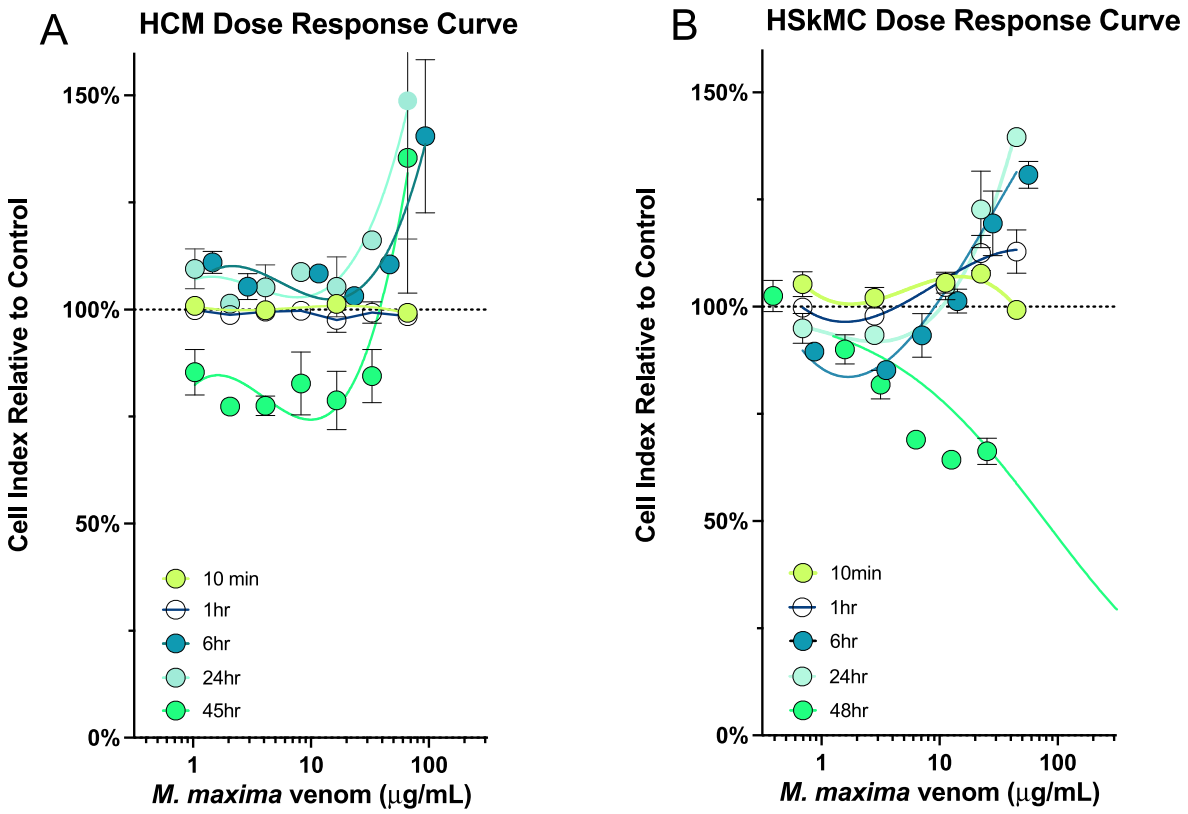

Fig. 5. Comparison of cell line response to *M. maxima* venom. Dose Response Curves, shown on x-axis, antilog, of *M. maxima* venom and cell index relative to control for **(A)** Human Cardiomyocytes (HCMs) and **(B)** Human Skeletal Muscle cells (HSkMCs) generated for the time course the cells were exposed to venom. Error bars on each data point indicate standard deviation of the mean, (n = 3). Some error bars are not visible as they are smaller than the symbol, and some points have been nudged (± 0.5 on y-axis) to aid data visualization.

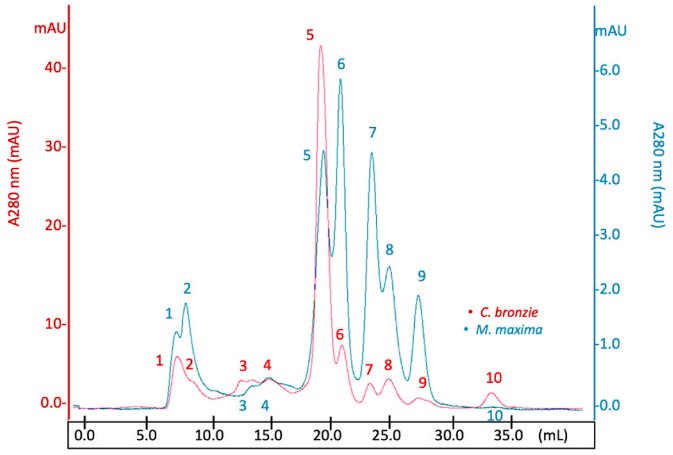

Fig. 6. Superdex 200 size exclusion column comparison of *C. bronzie* and *M. maxima* venom traces. Chromatogram of the venom of the two species show distinct absorbance profiles at 280 nm absorbance plotted on vertical axis against the eluted volume on horizontal axis. Each y-axis was scaled to the respective venom sample. The salt peak is at 21.8 mL, approximately peak 6, and is indicative of the salt peak and the end of conventionally separated proteins (Housley et al., 2020).

of rat isolated aorta (Winter et al., 2007). Treatment with prazosin, box jellyfish antivenom, long-acting ion channel blockers, muscarinic receptor antagonist or bradykinin receptor antagonist did not attenuate the effect of *C. bronzie* toxin on vascular response in rats (Winter et al., 2007). Future studies may consider testing *C. bronzie* venom on other subunits of voltage-gated calcium ion channel families, proteomic and genomic methods to further elucidate the mechanisms driving cardiovascular symptoms and cytotoxicity.

Several Carybdeida jellyfish species have been linked to the severe Irukandji syndrome witnessed in human envenomation (Tibballs et al., 2012; Carrette et al., 2012). Mechanistic knowledge contributing to the development of treatment for Irukandji Syndrome however, has yet to be elucidated (Tibballs et al., 2012). However, high levels of serum catecholamines have been suspected and proposed as part of the mechanism at work in human envenomation (Fenner et al., 1988; Martin and Audley, 1990). Elevated catecholamines have also been linked to Carybdeida venom challenge in animal models (Tibballs et al., 2001). This study shows for the first time that *M. maxima* venom is cytotoxic to HSkMCs after a long exposure (48 h). Furthermore, a 40–50% HSkMC index reduction occurred at 11.23 µg/mL compared to healthy controls. Slower rates of cell index reduction showed that *C. bronzie* venom is less toxic in our assay than *C. fleckeri* venom (Brinkman and Burnell, 2007), it is possible this may be a difference of mechanism between the two venoms (Fig. 2). *M. maxima* venom caused an initial rapid, sustained increase in cell index over time and at the higher concentrations in HCMs. Previous work has shown an increased cell index when cells were challenged with viviparous snake venom (Neale et al., 2018). *Carukia barnesi* venom has also been shown to cause increased cellular metabolism (Pereira and Seymour, 2013). Due to the short timeframe of these large increases in cell index produced by *M. maxima*, it is unlikely that the cells were proliferating and rather another activity was being detected. This increase in cell index could be due to the cells increasing in size (surface area) or adhering to the well bottom more tightly, both of which can occur in xCELLigence experiments (Xing et al., 2006). However, given the correlation between concentration and increasing cell index, mechanistic explanations driving cell changes, such as increased metabolism or ion channel activation, are also plausible. Future research

should investigate mechanisms that may be conserved in Carybdeida species for the development of novel, effective treatment modalities.

Notably, this study showed that HCMs had distinct higher cell index than the HSkMCs when exposed to *M. maxima* venom. Sustained increased cell index, also seen in a previous sea-snake venom study (Neale et al., 2018), are more difficult to interpret. While the mechanism of *M. maxima* is still unknown, evidence of sodium channel activation has been observed in *C. fleckeri* and *C. barnesi* studies (Mustafa et al., 1995; Winkel et al., 2005). *Carukia barnesi* venom induced tachycardia, in both rat and guinea pig isolated tissues, and was concentration dependent; tachycardia was nearly entirely prevented with the pre-treatment of Tetrototoxin (TTX), a well-known sodium channel blocker. Similarly, *C. fleckeri* venoms' cardiotoxic effects in isolated cardiac tissue and/or organs has been nearly entirely attenuated with non-specific ion channel blockers and sodium depletion (Mustafa et al., 1995; Bailey et al., 2005). Sodium channel activation may have induced a significantly greater effect in the metabolism of the HCMs, compared to the HSkMCs, when challenged with *M. maxima* venom, due to high quantities of clustered sodium channels found in the intercalated disks of cardiomyocytes (Rohr, 2004). Intercalated disks are unique to cardiac myocyte tissue and enable unified contraction (Rohr, 2004). Animal studies have shown Cubozoa venom-modulated sodium conductance has been effectively blocked with TTX. From these results, future research should compare mechanisms of venom action by directly blocking the activation of various voltage-gated sodium channels for both *C. barnesi* and *M. maxima* venoms.

Another possible explanation for the increase in cell index with increased *M. maxima* venom concentration is an increase in cell metabolism. Neale et al. found a similar increased cell index, in both HCMs and HSkMCs, with increasing concentration when challenged with four different viviparous sea snake venoms (Neale et al., 2018). The authors additionally showed the rise in cell index correlated with increased mitochondrial activity (Neale et al., 2018). The present study observed a continuous increase of cell index in HCMs in response to increasing *M. maxima* venom concentrations (Fig. 5A). Increased cell index was observed in HSkMCs, however, long exposure (>24 h) to the venom resulted in cell death. Inferring from Neal et al., the notably higher increase in cell index in HCMs compared to HSkMCs may be due to the higher density of mitochondria in HCMs (Rohr, 2004; Hall and Guyton, 2011). Induction of toxicity from metabolism stimulation is an intriguing potential mechanism and holds great interest for future studies.

5. Conclusion

In conclusion, this study utilized the xCELLigence system, a cell proliferation assay, to assess the response of two cubozoan venoms on HCMs and HSkMCs. Both *Chiropsella bronzie* and *Malo maxima* venom reduced the cell index, a measure of viability. While both venoms were found to be cytotoxic *M. maxima* venom showed a notable initial concentration-dependent increase in cell index. Future studies should further analyze the underlying mechanisms of cytotoxicity and increased cell index. Cubozoan envenoming can be fatal, and present a major cost in terms of public health, leisure and tourism. Treatments are needed that target the venom's mechanisms rather than development of antibodies, which have low efficacy. Novel mechanisms that target venom mechanisms common between multiple Irukandji-causing species are needed. Elucidating mechanistic action of these venoms will contribute efficacious, novel treatment modalities for envenomation and potential for venom derived pharmaceuticals.

Contributions

Melissa Piontek: Writing Original Draft & Reviewing and Editing, Conceptualization, Methodology, Formal Analysis, Validation, Investigation, Resources, Data Curation, Visualization, Project Administration, Funding Acquisition.

Athena Andreosso: Supervision, Methodology, Writing Reviewing and Editing.

Michael Smout: Supervision, Conceptualization, Methodology, Project Administration, Visualization, Data Curation, Resources, Formal Analysis, Writing Reviewing and Editing, Funding Acquisition.

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Ethical statement

The study did not involve animal or human subjects.

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.

Data availability

Data will be made available on request.

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