UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS: FISIOLOGIA ANIMAL COMPARADA

TOXINAS ENCONTRADAS NA ANÊMONA *BUNODOSOMA CANGICUM* CAPAZES DE CAUSAR MORTE CELULAR EM HEPATÓCITOS DE *Danio rerio* EM CULTURA.

CLAUDIO LUIS QUARESMA BASTOS JUNIOR

Orientador: Prof. Dr. Robert Tew Boyle

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Fisiológicas, Fisiologia Animal Comparada da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de Mestre.

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“Se você encontrar um caminho sem obstáculos, ele provavelmente não levará a lugar algum.”

J.F. Rozza #CDH

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Sumário:

RESUMO:

As substâncias bioativas presentes nos animais funcionam como venenos ou toxinas. Durante muito tempo o envenenamento através de toxinas oriundas de animais sempre foi alvo de muitas observações. As substâncias que tem propriedades de atuarem como ferramentas fisiológicas e/ou farmacológicas e trazem algum tipo de benefício à saúde humana, tendem a receber maior atenção. Uma análise dos compostos bioativos oriundos de animais marinhos mostra que a maioria é proveniente de quatro grupos: macroalgas, celenterados, equinodermos e esponjas. Dentre esses grupos, os celenterados ou cnidários, podem ser considerados potenciais fontes de biofármacos como, por exemplo, corais que produzem agentes antibióticos e anêmonas marinhas cujas toxinas agem em canais iônicos. O objetivo deste presente trabalho foi verificar o potencial que as toxinas extraídas de diferentes estruturas das anêmonas possuem em induzir a apoptose de hepatócitos de *zebrafish* (ZFL). Com este trabalho esclarecemos aspectos importantes da fisiologia destes animais. O fato de a sua toxina ter capacidade de causar apoptose, até então não havia sido evidenciado. Juntamente com isso nosso trabalho mostra substâncias naturais capazes de causar apoptose, fato que é de extrema relevância para as pesquisas futuras em diversas áreas do conhecimento. Anêmonas foram coletadas nos Molhes da Barra, após serem aclimatados no laboratório por uma semana, passou-se para os experimentos. Em nossos testes, utilizamos uma nova metodologia para obtenção de veneno nos fornecendo uma toxina mais pura. Foram realizadas extração de subprodutos de regiões da coluna e dos tentáculos das anêmonas, após as mesmas foram pipetadas sobre células de *zebrafish* em cultura que ficaram em exposição durante 60 minutos. Logo após analisou-se a morte celular via apoptose em um microscópio invertido de fluorescência. Os resultados mostram que as toxinas encontradas na coluna da anêmona possuem capacidade em causar apoptose em hepatócitos de *zebrafish*. Estas toxinas estão armazenadas em regiões específicas e não em todo o corpo do animal. Os subprodutos extraídos dos tentáculos não causaram apoptose. Até então não haviam sido levantados dados sobre toxinas da anêmona capazes de causar apoptose. Através da análise morfológica das células foi possível observar sinais característicos de apoptose celular. Este estudo mostra que mesmo após a purificação das toxinas as mesmas são capazes de causar efeito.

Palavras Chave: Apoptose, Toxinas, Bunodosoma cangicum, Vesículas.

INTRODUÇÃO GERAL:

Os Cnidários são organismos simples que possuem notáveis características fisiológicas suscetíveis a investigação microscópica, estão relacionados aos corais, águas-vivas, hidras e anêmonas. Estes animais não possuem cabeça, as suas extremidades são descritas como "bucal" (o mais próximo da boca) e "aboral" (mais afastada da boca), possuem simetria radial primária, tentáculos, estruturas urticantes ou adesivas (cnidas), cavidade gastrovascular incompleta como única cavidade corpórea e uma mesogléia (camada intermediária). Estes animais foram os **primeiros a apresentar uma cavidade digestiva no corpo**, também foram os primeiros a apresentar tecidos verdadeiros embora ainda não possuam a formação de órgãos. Os cnidários carecem de cefalização, seu sistema nervoso é uma rede nervosa simples, composta por neurônios nus e não polares e também não possuem estruturas individualizadas para trocas gasosas, excreção ou circulação; estes animais realizam movimentos de contração e de extensão do corpo, além de poderem apresentar deslocamentos (BRUSCA & BRUSCA, 2007). As anêmonas estão entre os representantes mais conhecidos do grupo estes animais são organismos solitários que se alimentam de pequenas presas, também podem absorver matéria orgânica dissolvida, e podem possuir relações endossimbiótica com outros animais. As anêmonas são encontradas na forma polipoide, habitam diferentes ambientes marinhos (BARNES, 1984).

Dentre as espécies de anêmonas, *Bunodosoma cangicum* é uma das mais abundantes na costa brasileira (ZAMPONI et al., 1998), sua coloração parda permite com que ela se esconda no substrato onde vive, e assim consegue proteger-se de predadores. A maior parte do seu corpo é formada por uma coluna, em suas extremidades possui um disco podal para adesão e um disco oral onde possui alguns tentáculos, no centro da cavidade encontra-se uma boca (BARNES, 1984) (Figura1). Os tentáculos das anêmonas são utilizados para capturar presas, estas estruturas possuem receptores químicos e sensoriais que ao perceberem a presa eles a capturam e jogam para dentro da cavidade digestiva, nesta região ocorre o processo de digestão, após os dejetos são liberados pela cavidade, e os tentáculos ficam novamente expostos a espera de uma outra presa.

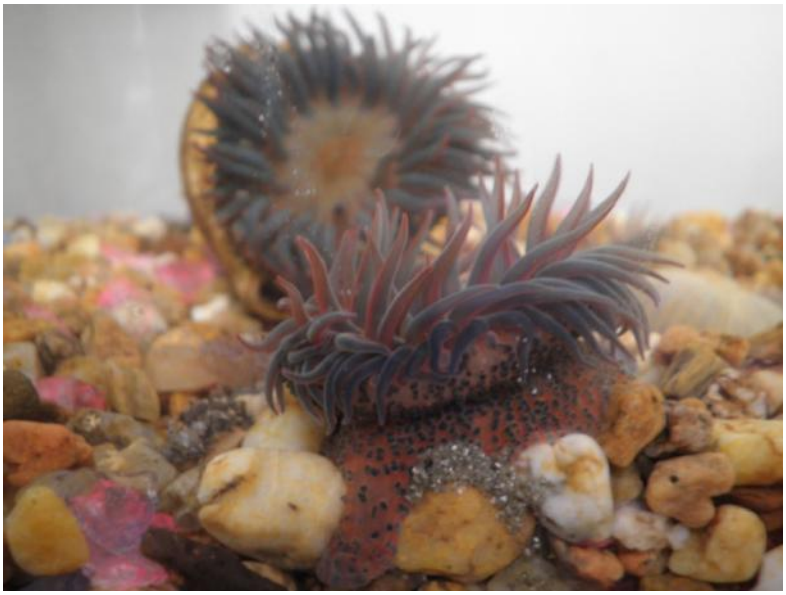

Figura 1 - Anêmona *Bunodosoma cangicum* com seus tentáculos expostos. (Fonte: Claudio Bastos Junior ICB-FURG)

Substâncias bioativas presentes nos animais funcionam como venenos ou toxinas. Durante muito tempo o envenenamento através de toxinas oriundas de

animais sempre foi alvo de muitas observações. Essas substâncias contribuem para o desenvolvimento de estudos fisiológicos e farmacológicos que permite o estudo da interação dessas substâncias com canais iônicos e vias metabólicas. As substâncias que possuem propriedades de atuarem como ferramentas fisiológicas e/ou farmacológicas e trazem algum tipo de benefício a saúde humana, tendem a receber maior atenção (IRELAND et al., 1993). Dentre os grupos de animais que apresentam vida apenas em ambientes aquáticos, os cnidários são o primeiro grupo de animais peçonhentos. As anêmonas assim como outros cnidários possuem cnidócitos que são células especializadas localizadas principalmente em seus tentáculos. Os cnidócitos podem conter toxinas que são injetadas na presa. Basicamente os cnidócitos são divididos entre 3 grupos: Espirocisto, Pticocisto e Nematocisto; Os espirocistos não liberam toxinas quando são disparados, possuem um filamento em forma de mola e filamentos menores pegajosos; Os Pticocistos também não liberam toxinas, porém seus filamentos menores não são adesivos e os Nematocistos os filamentos possuem espinhos que podem penetrar na presa, e ao serem evertidos liberam um coquetel de toxinas (NORTON, 1991; MINAGAWA et al., 1998). Cnidócitos do tipo nematocistos são os mais estudados e estes possuem potentes toxinas (KARALLIEDDE, 1995). Nematocistos possuem um formato de túbulo oco que ao explodir libera um arpão, esta explosão se dá quando a célula sofre algum tipo de estímulo. Ao ocorrer o disparo um coquetel de toxinas é liberado (SPADA, LA et al., 2002). Estudos envolvendo as toxinas de anêmona, relatam que as mesmas são de origem protéicas. Estes polipeptídios podem atuar como neurotoxinas, hemolisinas e fosfolipases podendo vir a gerar respostas patológicas como necrose e

eritema estas toxinas podem atuar em vertebrados ou invertebrados (SHER et al., 2005; ZAHARENKO et al., 2008). Ao consultar a literatura, são observadas abordagens com o efeito de toxinas das anêmonas sobre canais iônicos, e também efeitos de necrose e neurotoxicidade, porém não são relatados ensaios que visualizam o potencial destas toxinas em causar apoptose.

Outro fator que deve ser considerado é que nenhum experimento mostra a origem dessas toxinas nesse animal. A maior parte dos estudos envolvendo toxinas de anêmonas utilizam estímulos elétricos no animal para descarregar os nematocistos (MALPEZZI et al., 1993) ou então o animal inteiro é processado em um mixer (HONMA et al., 2005). Estas metodologias fazem com que ocorra uma mistura dos subprodutos provenientes de diferentes estruturas do animal. Dados da literatura (BIGGER, 1980), sugerem que as anêmonas possuem diferentes toxinas armazenadas em diferentes estruturas como, por exemplo, as estruturas denominadas de *acrorhagi*, as toxinas produzidas por essas regiões são diferentes das toxinas encontradas nos restante do corpo. Este fato justifica separar os subprodutos extraídos de cada região para testar os seus efeitos em separado.

Os *acrorhagi* localizam-se abaixo da coroa de tentáculos, são órgãos agressivos e geralmente utilizados em disputas territoriais com outras anêmonas da mesma espécie por isso os nematocistos encontrados nestas regiões são capazes de causar efeito em organismos co-específicos (BIGGER, 1980). Os organismos venenosos podem possuir resistências as suas próprias toxinas, as anêmonas utilizam os *acrorhagi* em seus conflitos co-especificos, ao contrário dos cnidócitos encontrados em outras partes do corpo, que possuem diferentes características,

estes encontrados em órgãos especializados são usados para disputas com anêmonas da mesma espécie por local, alimentação, luminosidade, etc. sendo visíveis apenas em momentos de luta (BARTOSZ et al., 2008)

Estes *Acrorhagi* possuem um formato de bolsas, que podem ser dilatados por movimentos de coluna, sendo posta em contato com outra anêmona invasora, aderindo-se ao corpo da vitima e descarregando nela os grandes nematocistos *holotrichous* que são diferentes dos nematocistos encontrados no restante do corpo, espera-se que eles contenham estrutura e/ou função de novas toxinas peptídicas (SHIOMI, 2009) (Figura 2).

Figura 2 - Em destaque pode se observar os *acrorhagi* postos em contato com outra anêmona invasora. (Fonte: http://www.arkive.org/beadlet-anemone/actinia-equina/image-A13068.html)

Os túbulos dos nematocistos *holotrichous* ancoram da ectoderme acrorhagial para o tecido alvo de modo que quando o *acrorhagi* é retirado aproximadamente 90% do ectoderme da ponta acrorhagial, permanece ligado a anêmona-alvo, esta concentrada descarga é capaz de provocar uma mancha de necrose no tecido onde ocorreu o toque com o *acrorhagi* (BARTOSZ et al., 2008)(Figura 3).

Figura 3 - Manchas de necrose no tecido onde ocorreu o toque com os *acrorhagi*. (Fonte: http://www.arkive.org/beadlet-anemone/actinia-equina/image-A13064.html)

Ao observar a morfologia da anêmona *Bunodosoma cangicum*, podemos notar que ao longo de sua coluna, são encontradas algumas estruturas com formato arredondado, a estas estruturas é dado o nome de vesículas (Fígura 4A e 4B).

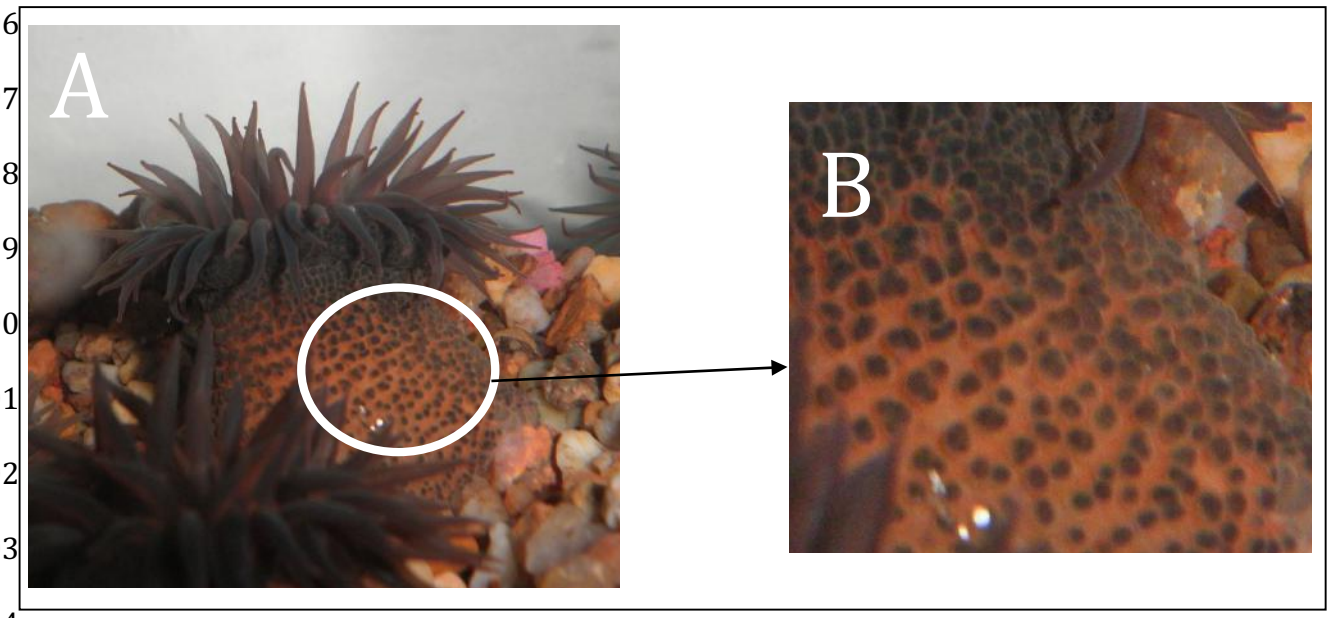

Figura 4 – Anêmona *Bunodosoma cangicum* com sua coroa de tentáculos exposta logo abaixo na coluna localizam-se as vesículas. A) Vesículas dispostas ao longo da coluna da anêmona, destacada com um círculo. B) Ampliação das vesículas da anêmona onde pode-se observar o formato arredondado das estruturas. (Fonte: Claudio Bastos ICB/FURG).

Poucos dados sobre estas estruturas são encontrados na literatura; Segundo DALY (2004) é comum encontrar em membros da família *actinidae* estruturas denominadas de verrugas ou vesículas dispostas ao longo de sua coluna. Ainda este mesmo trabalho relata que nas anêmonas do gênero *Bunodosoma* estas estruturas possuem características arredondadas e tipicamente não adesivas. Segundo este autor as mesmas não possuem nematocistos em suas estruturas. Porém, em verdade, poucos são os pesquisadores que dão importância a estas estruturas tendo em vista o que já abordamos sobre testes com a mistura dos compostos bioativos gerados pelo animal. Esta mistura apenas mostra que o animal tem toxina, não mostrando realmente os locais que podem liberar esta toxina, ou no entanto armazenar esta toxina. Estudos utilizando apenas subprodutos destas regiões não foram realizados anteriormente. Nosso grupo é o pioneiro a realizar testes com subprodutos extraídos exclusivamente desta região e também o primeiro grupo a

evidenciar potencial apoptótico que substâncias extraídas unicamente dessas regiões possuem.

O modelo biológico para os testes realizados por nós foram células de hepatócito de zebrafish em cultura (ZFL-Cells), estas células são de uma cultura estabelecida e são de uma linhagem normal. O parâmetro analisado por nós foi a morte celular programada (apoptose). A apoptose pode ser induzida através de fatores intracelulares ou extracelulares, este mecanismo faz com que ocorra uma eliminação das células que sofreram algum dano. Esta eliminação faz com que se mantenha a integridade do organismo como um todo. Este processo requer gastos de energia já que durante a apoptose a célula sofre toda uma transformação até a formação dos corpos apoptóticos, ao contrário da necrose que não requer gastos de energia (GRIVICICH et al., 2007).

Diante de todas as informações acima, nesta dissertação se verificou o potencial que toxinas provenientes da anêmona em suas diferentes localizações têm em causar apoptose. Nosso grupo utilizou uma metodologia para obter um veneno mais puro, e também somos os primeiros a relatar dados de toxinas encontradas exclusivamente na coluna do animal, e ainda os primeiros a relatar que essas toxinas possuem um forte potencial de causar apoptose.

Com base nos dados levantados, o objetivo deste trabalho foi verificar o potencial de causar morte celular que toxinas presentes na coluna da anêmona *Bunodosoma cangicum* possuem.

Polypeptides secreted from the columnar vesicles of the anemone *Bunodosoma cangicum* induce apotosis in the ZF-L zebra fish cell line.

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Key words: apoptosis, anemone venom, columnar vesicles, zebrafish cell line

Abstract

We show that the stimulated secretion a mixture of polypeptides from the columnar vesicles of the anemone *Bunodosoma cangicum* induces apoptosis in the zebrafish cell line, ZF-L. The active peptides are heat labile, react with ninhydrin, and are weakly stained with Coomassie R50. Gel filtration chromatography indicates that the venom mixture probably contains more than one active form. Microscopic and flow cytometric assays confirm the apoptotic activity. PAGE analysis shows the venom only contains low weight peptides and no significant protein. This manuscript is the first report of biologically active peptides associated with columnar vesicles of *B. cangicum*.

Introduction

The Cnidarian phylum is diverse, including jellyfish, coral, hydras and anemones. They represent the earliest animals with a digestive cavity, true tissues but not organs, and were also the first to develop a nervous system in the form of a neral net (R. C. Brusca & Brusca, 2007). Common to animals of this phylum is a radial symmetric body plan, and most notably, the presence of tentacles surrounding the oral cavity, which house batteries of stinging cells, nematocytes. (R. C. Brusca & Brusca, 2007) All Cnidaria have cnidocytes; of which nematocytes and spirocytes are specialized subsets. Cnidaria are also typically venomous, and it is the nematocytes, through the specialized organelle, the nematocyst, which delivers the venom-injecting lancet when the tentacles make contact with prey or predator. As such, this venom delivery system can serve both predatory and defensive roles. The venom is often a complex mixture of proteins and peptides which can exhibit an array of highly specific biological activities. For example, anemone venom is known to contain cytolytic proteins and peptides, phosphatase enzymes, and elegantly specific membrane channel-blockers (Bosmans & Tytgat, 2007; Diochot, Schweitz, Béress, & Lazdunski, 1998; Oliveira et al., 2004; Zaharenko et al., 2008). All of these components may work in concert to debilitate small vertebrates or invertebrates that make physical contact with the anemone tentacles.

However, it has also been known for many decades that some cnidocytes may not contain venom, for example, a different subset of cnidocytes, termed spirocytes, contain a sticky lancet which serves to entangle and immobilize potential prey

without penetration or intoxication (Fautin, 2009) (Doumenc, 1971).

Bunodosoma cangicum, an anemone that we here show to exhibit spirocytes as their principle tentacular cnidocyte, is also known to be nonetheless venomous, and there are several reports characterizing the venom obtainable from *B. cangicum*. (Oliveira et al., 2006; Wanke, Zaharenko, Redaelli, & Schiavon, 2009; Zaharenko et al., 2008) This apparent contradiction may be in part due to the fact that a single cnidarian can possess different subsets of cnidocytes and they may be arranged in non-homogeneous distribution patterns in both the tentacles and body column of the particular specie (Shick, 2012) (R. C. Brusca & Brusca, 2007) (Daly, 2015). In recent studies, immunocytochemical techniques have shown that venom is present in an ectodermal gland of an anemone tentacle in patches that are devoid of nematocytes. (Moran et al., 2012). So it seems that anatomical structures other than nematocytes may also deliver venom. Also, some anemones may also have specialized tentacle-like structures for interspecific aggression, termed acroraghi. These smaller tentacles lie beneath the principle catch tentacles, and may engorge, becoming more prominent during conflict between anemones. It may not be surprising that these acroraghi can contain nematocytes and venom profiles different from catch tentacles, and this has been shown to be true (Honma et al., 2005). Bartosz and collaborators have shown that acroraghial venom from *Actinia equina* induces necrosis at least in part through the generation of reactive oxygen species (Bartosz, Finkelshtein, Przygodzki, & Bsor, 2008). Also, recent reports have shown that anemone venom may cause cell death through apoptotic pathways. (Ramezanpour, da Silva, & Sanderson, 2014; 2013). Apart from these studies, little is known

regarding the mechanisms of cell death that are induced by anemone venom, even though it has been studied for many decades. The most common methods for extracting venom from anemones are simple but often require multiple chromatographic methods to purify the extract. For example, some researchers in the past have simply homogenized entire animals in blenders. It has also been common to electrocute whole animals, inducing massive cnidocyte discharge, to obtain a crude venom (Malpezzi, 1991). However, with these techniques, it is not possible to locate the precise source of the venom. A somewhat more precise method is to place the anemones in plastic bags and manually massage the tentacles to release venom, a process called "milking" (Nedosyko, Young, Edwards, & da Silva, 2014). An established technique for stimulating tentacular nematocyte discharge *in vitro* is through cellular depolarization via reversal of the Nernst potential for the potassium ion (Luxoro & Nassar-Gentina, 1984). Thus, an increase in extracellular K^+ to 70mM, induces cnidocyte discharge. Our group routinely uses this technique to test the effects of dissolved metals and pollutants on the discharge efficiency of *B. cangicum* tentacles *in vitro* (unpublished observation). We have chosen this method of venom extraction through cellular depolarization for our study; combined with careful dissection of *B. cangicum* to provide new insights into the physiology of this animal. The *Bunodosoma* genus of anemones is characterized by numerous dark warty patches covering the body column of the animal, denominated vesicles (Daly, 2015) and may be seen in Figure 1. It is these vesicles that we have isolated and induced to secrete a mixture of biologically active molecules, including polypeptides, which was then tested on cultured cells to determine the mechanism of the induced

cell death. The present study is novel in that we attribute the presence of apoptosis inducing low weight polypeptides to a specific anatomical structure on this anemone's column. The function of these structures remains unclear, but it is possible that they serve in a defensive capacity.

Methods

Animal collection

Anemones *B. cangicum* were collected from the sandy areas surrounding a concrete jetty (molhes da barra) in Cassino Beach, located in the city of Rio Grande, the state of Rio Grande do Sul, in Southern Brazil (32° 09 ' 40.25 '' S; 52° 05 ' 51.96 '' W). Collections were performed year-round and the anemones ranged in size from 2.5 to 5cm column length. The animals were subsequently transported to the laboratory and maintained in aquaria with natural seawater at 22°C with constant aeration and a light cycle of 12h light/12h dark. They were allowed to adapt to this environment for at least two weeks before experiment. The water was changed weekly and the animals were handfed small pieces of shrimp and fish.

Venom preparation

Samples of transected catch tentacles and freshly dissected columnar vesicles were placed in Eppendorf tubes with 0.5ml artificial seawater (ASW) (composition in mM: NaCl 399.71, KCl 8.82, $CaCl_2$ 8.82, $MgSO_4$ 45.88, $NaHCO_3$ 2.21, pH 7.8) at 4°C. Sample tubes of tentacles typically contained two pieces approximately 2-5mm in

length. Sample tubes containing vesicles typically contained five whole vesicles 2-4mm in diameter. The concentration of potassium ions in the ASW was then raised to 70mM by the addition of concentrated KCl. This K^+ augmentation induced a rapid and complete discharge of the tentacular cnidocytes. The columnar vesicles are visually absent of cnidocytes, and as such, no discharge was observed. After cellular depolarization, the anemone tissue was removed and the samples' supernatants were centrifuged at 17,000 G for 7min to sediment any tissue fragments and mucous. The supernatant of these samples was reserved as the crude venom and subsequently tested on cultured cells. The crude venom was also further purified by gel chromatography. Additional controls were performed in which tentacles and vesicles were placed in Eppendorf tubes with ASW, but without elevated K^+, to test for constitutive release of active peptides.

ZF-L Cell culture

The zebrafish hepatocyte cell line, ZF-L, is a non-transformed cell line obtained from the Cell Bank, Univ. Federal do Rio Janeiro. Cells were maintained in RPMI culture medium (Gibco/Life Technologies) supplemented with 5% fetal bovine serum (Gibco/Life Technologies) and 1% antibiotic/antimycotic (Gibco/Life Technologies). Cells were grown to confluence in 25cm^2 culture flasks and then sub-cultured into 24 well plates, 48 hours before experiment, using the same culture medium.

Venom exposure

Each well of 24-well plates, which contained confluent ZF-L cells in 1mL culture medium received 10µl of crude tentacle venom or columnar vesicle venom (each representing approximately 2% of each venom extraction batch). Other experiments used the same volume of purified vesicle venom fraction, or control solutions (ASW and ASW+ 70mM K^+). After various time periods, including: 30 min, 1h, and 2hs at room temperature, the microscopic assays were performed. Experimental groups contained sample wells with tentacle venom or vesicle venom, and control wells consisted of untreated ZF-L cells, cells treated with ASW alone and cells treated with ASW and elevated K^+.

Apoptosis/Necrosis detection by fluorescence

Mechanisms of cellular death were determined in part by the ethidium bromide/ acridine orange fluorescent assay (Sigma, St Louis, USA). Our analysis is derived from Kasibhatla et al. (Kasibhatla, 2006). After venom exposure, cultured cells were incubated in a mixture of 0.5 µg/ml acridine orange and 0.5 µg/ml ethidium bromide for 1 min. The labeling solution was replaced with 1ml PBS (phosphate buffered saline, pH 7.4) and the plate was immediately inspected by epifluorescence microscopy, using a standard fluorescein filter set (ex. 480nm, em. 525). Care was taken to precisely control the exposure times of the vital stains; thus the complete stain and wash procedure was repeated for every individual well of the plate to avoid false results that may arise from the toxicity of the stains.

Light Microscopy

All microscopy was performed with an Olympus IX81 inverted epi-fluorescence microscope. Images were captured with an Olympus DP72 CCD camera and post-processed with NIH ImageJ.

Gel filtration chromatography

To purify the crude vesicle venom extract, we performed size exclusion chromatography using a 10g Sephadex G-50 medium grain (Sigma) column at atmospheric pressure. The column was prepared in a 20ml syringe and equilibrated with phosphate buffered saline pH 7.4 at 4°C, then crude venom (0.5ml) was applied to the column. Thirty fractions were collected, each fraction containing 200µl of eluent. Fractionated venom was then applied to cultured cells using the same procedures developed for the crude venom.

Flow Cytometry

Individual venom fractions produced by gel filtration were incubated with ZF-L cells in suspension for 30 min. The cells were then tested with the Annexin V - FITC Apoptosis Detection Kit (Sigma) for the presence of phosphotidylserine in the outer cell membrane by flow cytometry, employing an Attune (Life Technologies) flow cytometer.

PAGE analysis

Crude venom plus four Sephadex column fractions showing apoptotic activity, and one fraction showing no activity, were subjected to polyacrylamide gel electrophoresis. Samples were electrophoresed using a 5% stacking gel and 7.5% resolving gel.

Total protein and peptide determination

Total protein was determined by a fluorescence based assay using the EZQ Protein Quantitation Kit (Life Technologies). The presence of peptides was estimated by ninhydrin reaction (Friedman, 2004).

Heat inactivation assay

Crude venom and chromatography column produced fractions were tested for heat resistance by pre-incubation in a 60°C water bath for 1h. The samples were then assayed as described in **Venom Exposure.**

Results

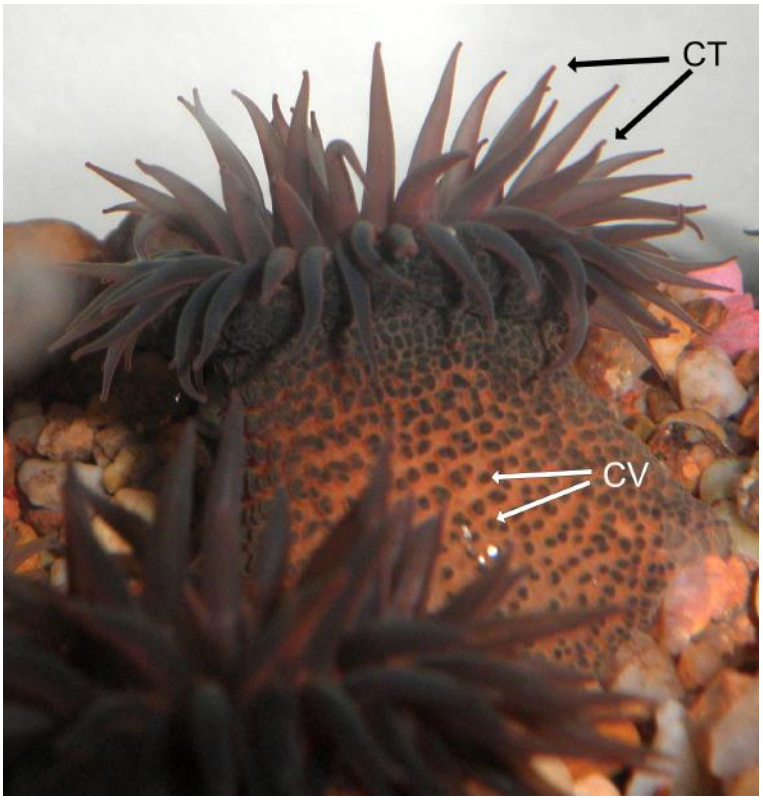


Figure 1- *Bunodosoma cangicum,* the anemone species employed in this study. Clearly apparent are the catch tentacles (CT) and the dark warty vesicles (CV) covering the animal's column.

The first assay employed in our study was a morphological and selective staining based fluorescence assay using a mixture of the nucleic acid reactive dyes, acridine orange and ethidium bromide. This assay was developed to broadly discriminate between necrosis and apoptosis cell death pathways (Mironova, Evstratova, & Antonov, 2007). Representative controls cells for this assay may be seen in Figure 2A, which is a phase contrast image of ZF-L cells (scale bar 100µm). Typical control cells when submitted to the acridine orange and ethidium bromide mixture and

viewed by fluorescence microscopy using a standard fluoroscene filter set, may be seen in Figure 2B. These are control ZF-L cells in the acridine orange/ethidium bromide assay, at a higher magnification (scale bar 50μm) to show individual cell nuclei. The homogenous, non-punctate, labeling of cell nuclei with acridine orange, seen in the image as green fluorescence, and the absence of any ethidium bromide staining, which produces a bright orange/red fluorescence, are morphological indicators of healthy cells with intact cell membranes. Our first treatment group used ZFL cells exposed to preparations of tentacle venom as described in the Material and Methods section. The results maybe seen in Figure 2C, which shows that ZF-L cells exposed to tentacle venom preparations appear morphologically normal after 1h, and their fluorescence profile is identical to Figure 1B control cells (not shown). Figure 2D, ZF-L cells in which macerated tentacle (approx. 1mg wet weight/ml) has been placed directly in the cell culture medium for 1h. Clearly visible are batteries of cnidocytes. The ZF-L cells in panel D are morphologically normal. The inset image in this panel shows a representative tentacular cnidocyte at higher magnification, confirming that they are spirocytes. Only spirocytes, and occasionally smaller cnidocytes, apparently desmonemes, have been observed in all tentacle preparations (more than twenty) of *B. cangicum* in our laboratory.

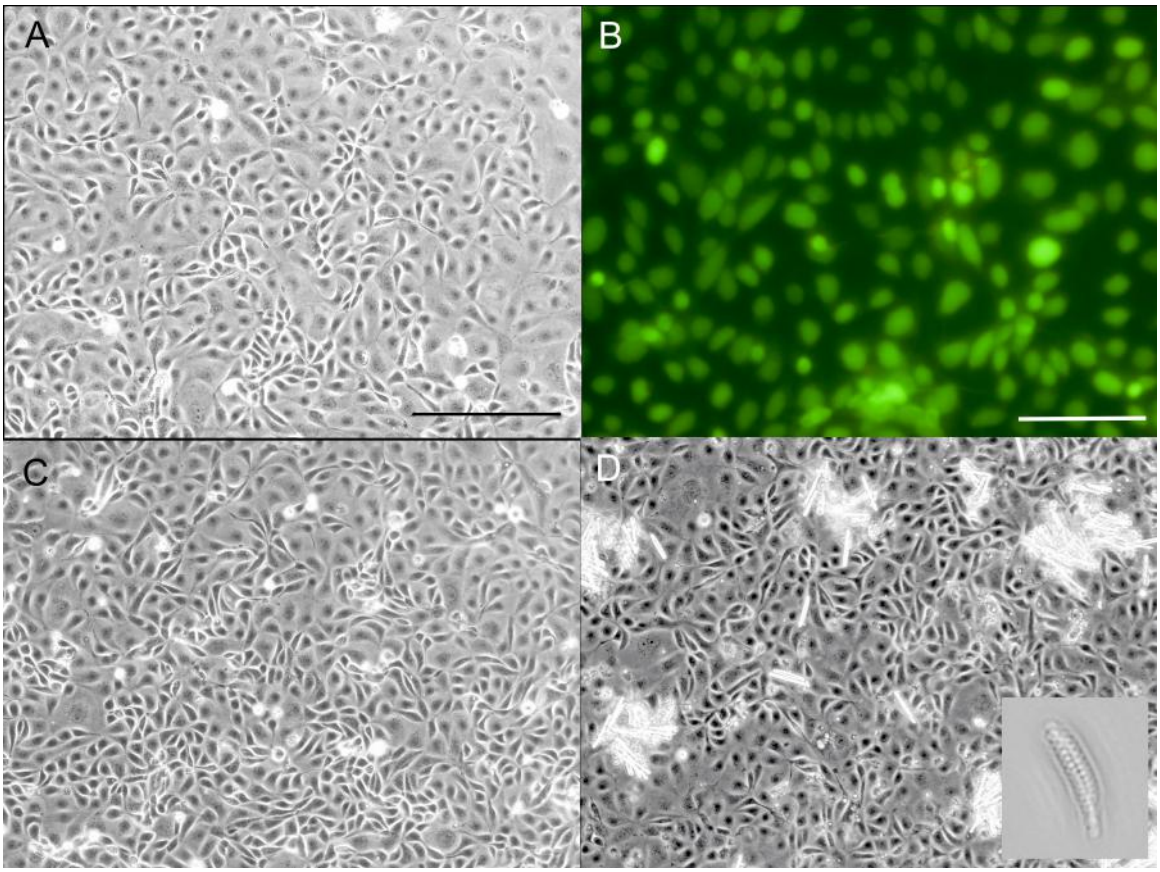
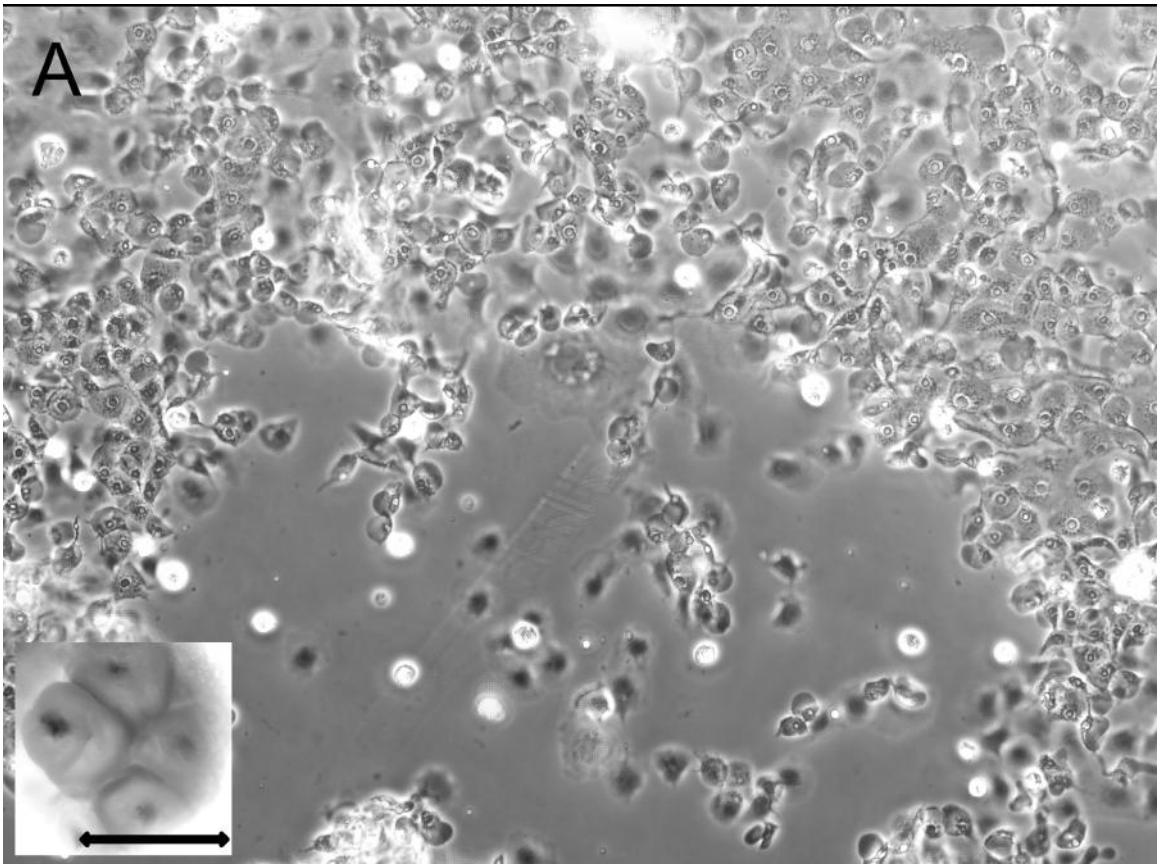


Figure 2 - Control ZF-L cells, scale bar 100µm (A). Control ZF-L cells exposed to acridine orange/ ethidium bromide assay solution, scale bar 50µm (B). ZF-L cells exposed to tentacular venom preparations for 1h, same scale as panel A (C). ZF-L cells exposed to macerated tentacles for 1h, same scale as panel A (D). The inset image in panel D shows tentacular cnidocytes to be spirocytes.

Figure 3, panel A shows the result of application of crude vesicle venom to the cultured cells after 1h. The cells show typical morphological characteristics associated with apoptosis, including: extensive membrane blebbing, pyknosis, and apoptotic bodies. The inset image in panel A shows typical columnar vesicles used in the venom extraction process. The scale bar for the inset is 1.5mm. Figure 3, panel B shows punctate staining of acridine orange (green), an indicator of pyknosis, and

512 the presence of very little ethidium bromide staining (bright orange/red). Figure 3,
513 panel C shows an overlay of panels A and B. The scale bar in panel C is 100 μm. The
514 double label experiment results indicate cell death via some type of apoptosis, since
515 the cell nuclei have condensed, the cell membranes have remained intact, and
516 apoptotic bodies are present, some containing nuclei acid, as shown by the intense
517 punctate ethidum bromide stain (orange/red spots).

518 

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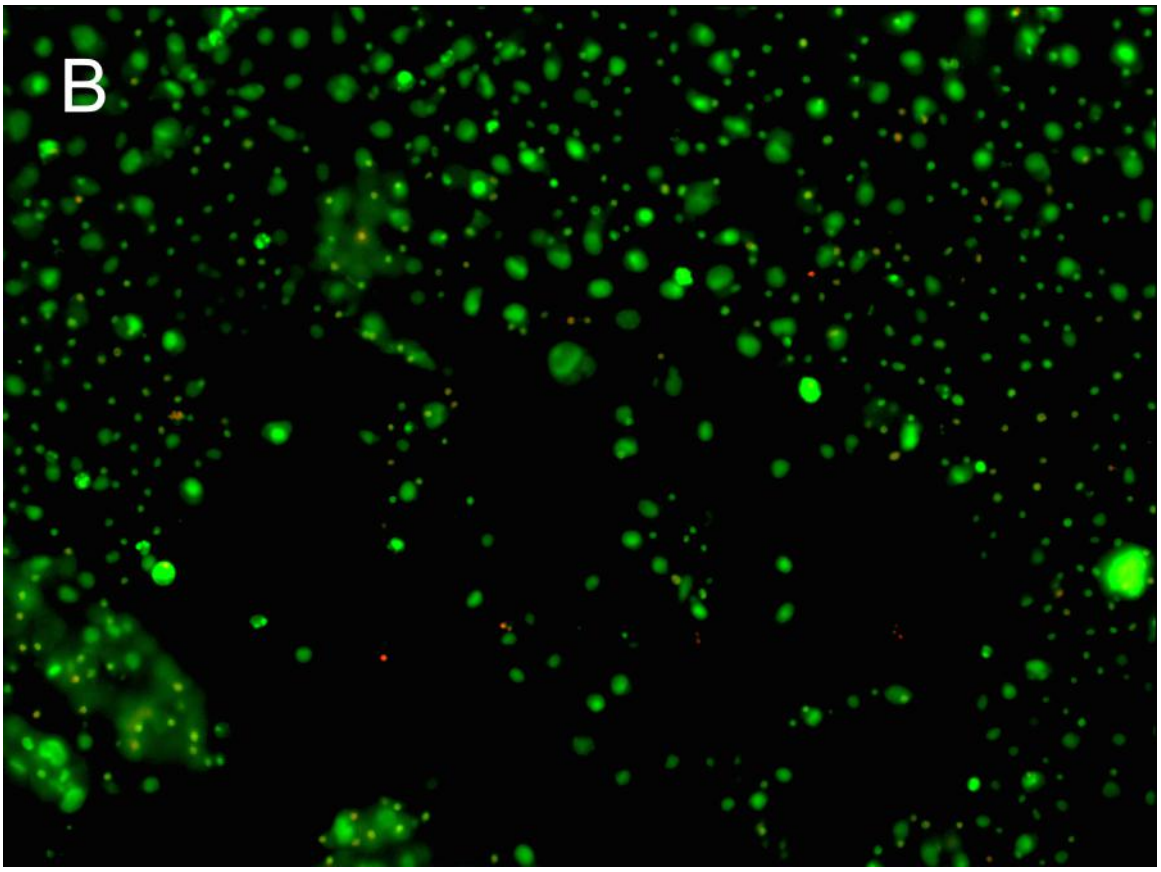

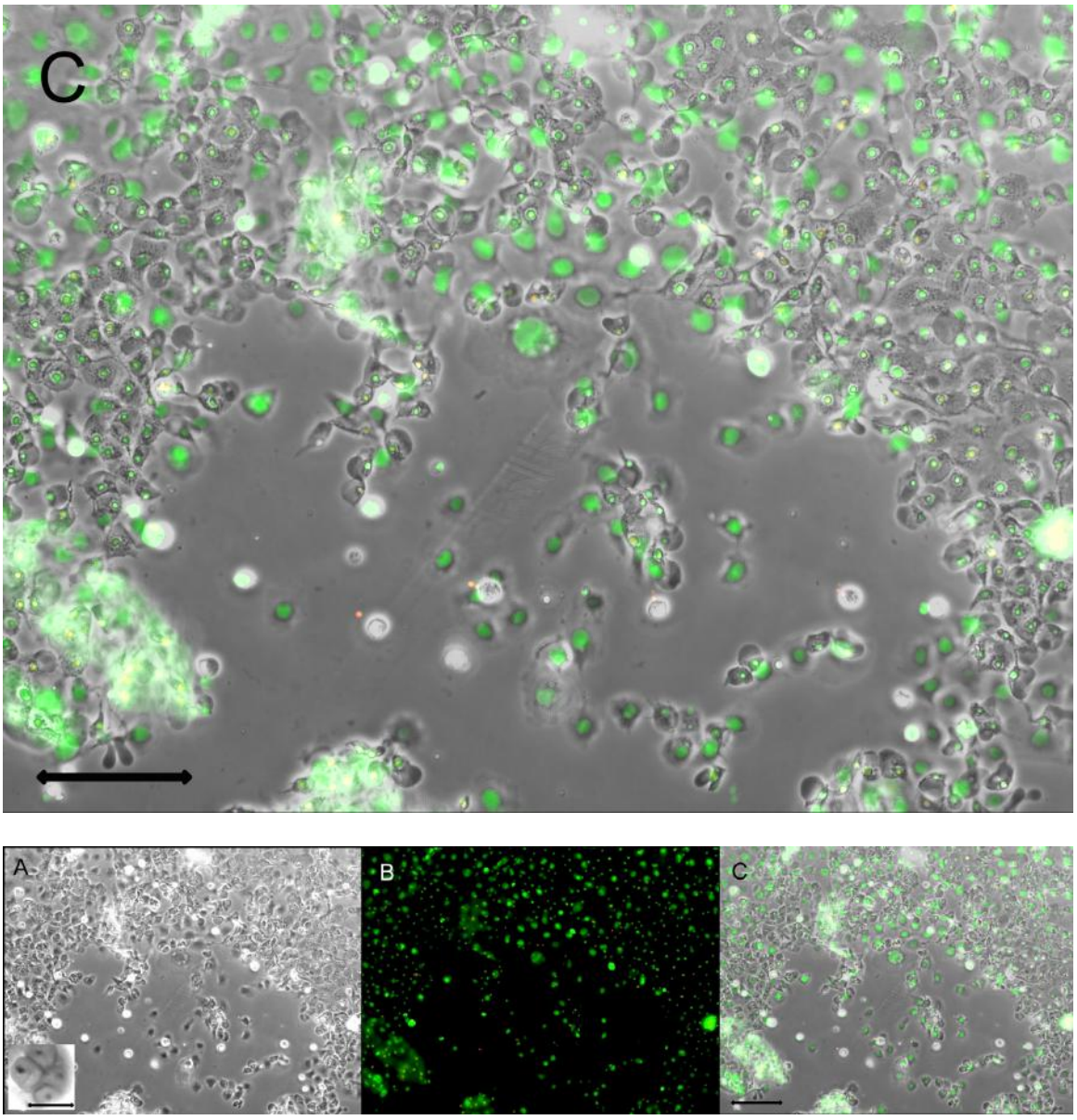

Figure 3 ZF-L cells exposed to crude vesicle venom for 1h (A). ZF-L cells, exposed to venom and labeled with acridine orange/ ethidium bromide solution (B). Overlay of panels A and B, scale bar 100µm. Inset image in panel A are representative columnar vesicles, scale bar 1.5mm.

After confirming that catch tentacles from *B. cangicum*, prepared by either cellular depolarization or through physical maceration, do not produce lethal effects in ZF-L

cultures, we decided to focus on the columnar vesicle preparations as they produced marked morphological disruptions of ZF-L, (Figure 3). Our crude vesicle venom was then separated into 30 equal fractions of 200µl by size exclusion chromatography, and these individual fractions were microscopically analyzed for their ability to reproduce the same morphological characters observed with crude venom in Figure 3. These results may be seen in Figure 4, A-D, which shows the morphological analysis of four representative vesicle venom fractions. Fractions tested were A (F15), B (F16), C (F22), and D (F27). The results are indistinguishable from crude venom effects (Figure 3).

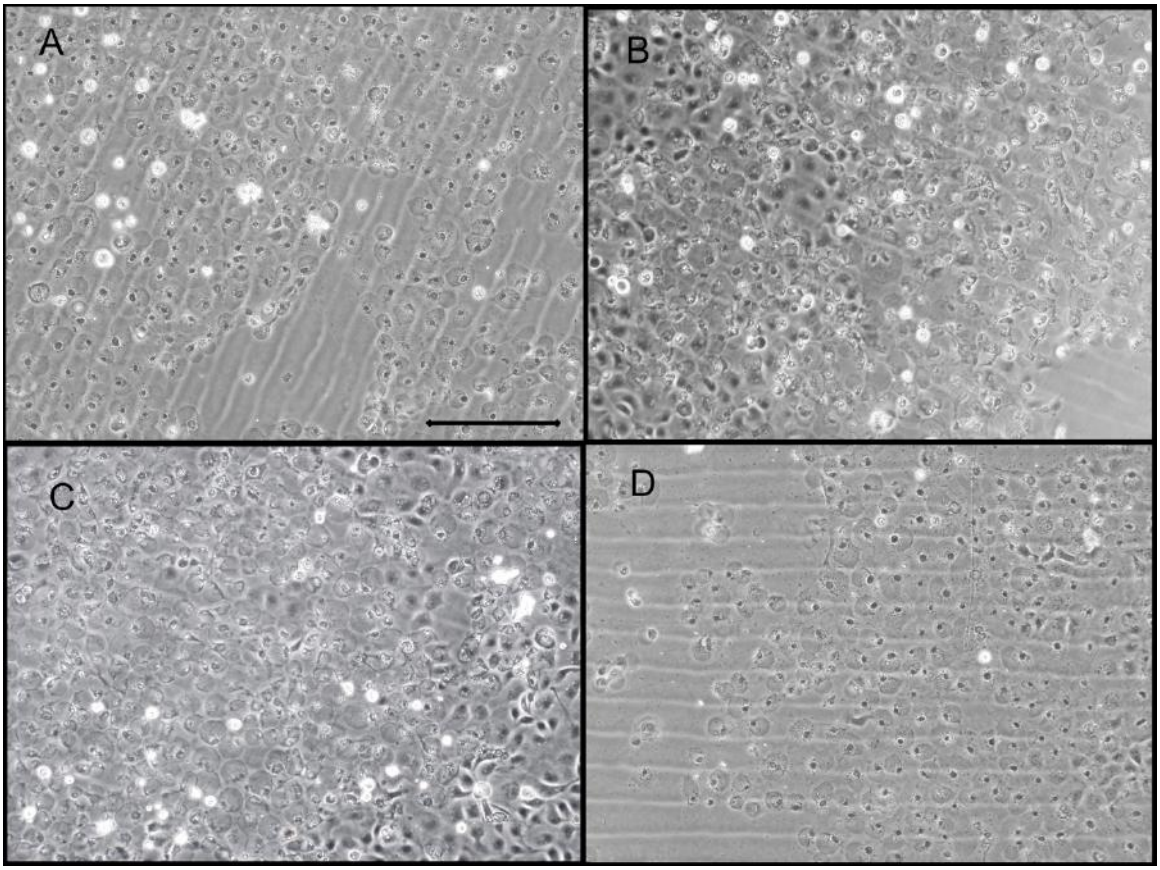

Figure 4 - ZF-L cells exposed to venom fraction 15 (A). ZF-L cells exposed to venom fraction 16 (B). ZF-L cells exposed to venom fraction 22 (C). ZF-L cells exposed to venom fraction (27). Scale bar 100µm.

In the early stages of apoptosis, the plasma membrane phospholipid, phosphotidylserine, flips from the inner leaflet plasma membrane to the outer leaflet where it may be detected by binding proteins and antibodies. Thus, in order to obtain corroborative evidence that the morphological changes in ZF-L that we were observing was indeed some form of apoptosis, we decided to test for the presence of phosphotidylserine in the exterior leaflet of the plasma membrane of ZF-L cells exposed to venom fractions, using a commercial kit which contains the protein, annexin 5A, which binds phosphotidylserine, together with an anti-annexin V - FITC labeled antibody. The presence of the phosphotidylserine/annexin/ anti-annexin antibody complex on the cell surface of apoptotic cells can then be detected by flow cytometry. Figure 5 shows the results of the flow cytometry analysis of various fractions of vesicle venom. Some contiguous venom fractions were combined for the analysis. The results indicate that combined fractions of vesicle venom from fraction number 17 (F17) and above, produce significant apoptosis. Higher fraction numbers from the gel filtration column should correspond to smaller peptides. These results suggest that vesicle venom may be a mixture of peptides of varying sizes.

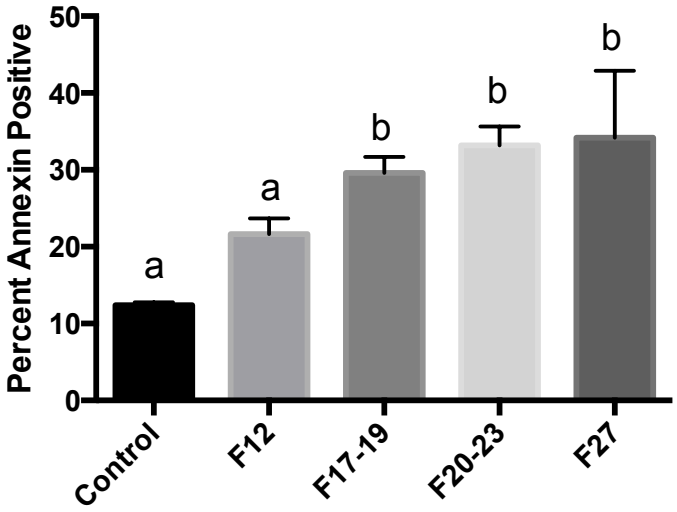

Figure 5 - Results of the Annexin V-FITC apoptosis detection kit via flow cytometry. Groups were compared by one-way ANOVA and columns were compared to control by Dunnett's post-test. Different letters indicate a significant difference, alpha = 0.05. Control N=5, F12 N=3, F17-19 N=4, F20-23 N=7, F27 N=2.

Next we wanted to know if our venom mixture is comprised of proteins, peptides or both. For this purpose, we performed a polyacrylamide gel electrophoresis of our crude venom and various venom fractions, the results are shown in Figure 6. The PAGE results show that vesicle venom is most likely a mixture of polypeptides of similar weight, but does not contain larger proteins. The Coomassie R50 stained bands are coincident with the bromophenol front, which indicates a negligible molecular weight.

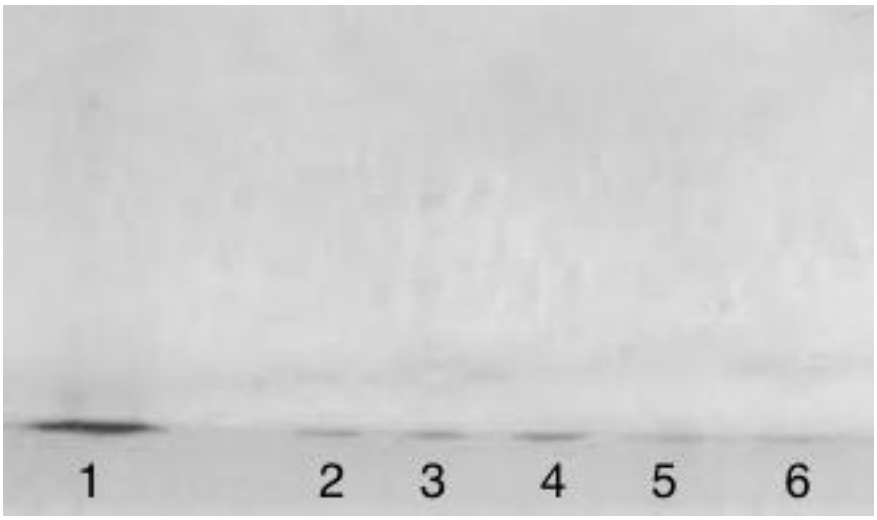

Figure 6 - PAGE analysis of crude vesicle venom, Lane 1, and various gel filtration column fractions. Fraction 5 (Lane 2), fraction 10 (Lane 3), fraction 17 (Lane 4), fraction 22 (Lane 5), fraction 28 (Lane 6).

Table 1 Shows the results of various assays to facilitate comparisons of fractions.

Table 1

Vesicle Venom Fraction Number	Protein by Flourescence Kit (μg/ml)	Ninhydrin Positive Reaction	Comassie R50 Positive Reaction (PAGE)	Apoptotic Activity (Microscope)	Annexin Positive (Flow Cytometry)	Heat Labile at 60 degrees Celsius (1h)
Pre-column	705	-	++	+	not tested	+
F5	-	-	+	-	not tested	not tested
F10	-	+	+	+	-	+
F17	240	-	+	+	+	+
F22	-	-	weak	+	+	+
F28	-	++	weak	+	+	+

Table 1 a comparison of various biochemical assays applied to the vesicle venom fractions.

Discussion

The recognition of the existence of programmed cell death, usually synonymous with apoptosis, is relatively recent and studies in the mechanisms involved in types of cell death have gained momentum since the first scientific descriptions of what Lockshin and Williams called programmed cell death, were reported in insects in 1965 (Lockshin & Williams, 1965). The landmark paper which brought us the term *apoptosis* and focused attention on this fundamental physiological process, is attributed to Kerr *et al*. in 1972 (Kerr, Wyllie, & Currie, 1972). In that year, this group published a series of human and rat histological sections, photographed by transmission electron microscopy, which defined the morphological characters of apoptosis used until today. The subsequent attention focused on the study of cell fate decisions such as apoptosis and necrosis, and their underlying mechanisms, is warranted. It is believed that perhaps half of all pathologies are affected by breakdowns in proper cell death regulation, including: cancers, autoimmune diseases, and neurodegenerative disorders and others (Fischer & Schulze-Osthoff, 2005).

When cells cease to exist, the routes to this end may be most broadly described as apoptosis, autophagy and necrosis. It is now more clear than within these three groups are, what Dawson et. al. refer to as various “subroutines”. (T. M. Dawson, Dawson, & El-Deiry, 2012). Attempts to subdivide apoptosis have revealed an

611 inherent problem; apoptosis is morphologically defined, but not biochemically
612 defined (Kroemer et al., 2005), and thus confusion can arise when researchers try to
613 diagnose apoptosis through biochemical assays. Nonetheless it is accepted that the
614 two major pathways of apoptosis are extrinsic and intrinsic, but other
615 denominations exist such as caspase dependent or caspase independent, and efforts
616 to describe types of apoptosis by its induction agents are popular, such as: redox
617 sensitive apoptosis and endoplasmic reticular stress induced apoptosis. The lexicon
618 of terms for specific types of apoptosis is growing, cornification is apoptosis in
619 keratinocytes and anoikis is apoptosis induced by cellular detachment from its
620 substrate; for a review of apoptosis related nomenclature, see Kroemer et al., 2005,
621 2008 and Dawson et al., 2012 (Kroemer et al., 2005) (Kroemer et al., 2008) (T. M.
622 Dawson et al., 2012).
623
624 We used two complementary microscopic evaluations and flow cytometry to
625 indicate the mechanism of cellular death of ZFL cells in response to the *B. cangicum*
626 peptides. Our assays, though commonly applied in research and well represented in
627 the literature, can only indicate in what is quite ample terms, apoptosis and
628 necrosis. There is of course, accumulating evidence that both necrosis and apoptosis
629 are multifaceted and not even mutually exclusive (Chipuk & Green, 2005). It now
630 seems that cells, once dedicated to self-destruct, can even switch between modes
631 and mechanisms of cell death if a preferential pathway is inhibited (Maiuri et al.,
632 2007). In our fluorescence microscopic assay, we used a co-exposure of equal
633 concentrations of acridine orange and ethidium bromide to aid in the morphological

evaluation of the mechanisms of cell death, broadly defined as apoptosis or necrosis. Both dyes stain DNA, however acridine orange is cell permeant while ethidium bromide is not. Therefore, normal healthy cells will show green nuclei with homogenous staining, as can be seen in Figure 2B. What is interesting about this figure is that most nuclei in the panel have a consistent level of fluorescence, with the exception of four or five cells with noticeably brighter nuclei. We believe these cells to be pre-mitotic and contain more DNA, producing a higher signal of acridine orange. There is no ethidium bromide signal in Figure 2B, which would appear as orange/red fluorescence. The appearance of this signal would indicate a compromised plasma membrane in the cultured cells, a hallmark of necrosis. In Figure 3 one may see a different character of acridine orange staining, the nuclei of the affected cells have shrunken, producing a punctate, bright green fluorescence. The shrunken (pyknotic) nucleus is an indicator of apoptosis and the fact that the fluorescence is green indicates that ethidium bromide has been excluded and thus the plasma membranes are intact; another indicator of apoptosis. The ethidium bromide has stained small apoptotic bodies in figures 3B and 3C, which appears as bright orange/red spots.

It is also interesting that various fractions from our Sephadex column separation obtained in our study show the ability to induce apotosis in ZF-L. It has yet to be confirmed that these fractions are truly different in terms of their composition. We believe however, that there are at least some compositional differences among the fractions as we typically see 2-3 fractions between F12 and F15, which have

apoptotic activity, after which, 2-3 fractions elute that show no activity, and then the activity returns around F18 and continues to above fraction F25, so we believe the crude venom is heterogeneous and that the column is not simply diluting a homogeneous venom. Of course, it must also be considered that a fraction collected in one venom processing procedure may not be equivalent to the same fraction number produced on a different day, some variability, at this point in our studies, must be expected. In considering the question of the compositional variability of these fractions, it is helpful to consult Table 1. Here we may see that the fractions indeed deviate in their responses to different biochemical assays. Notably, fraction F28 stained only weakly with Coomassie R50, showed no detectable protein in the fluorescence kit assay, but gave the strongest signal in the ninhydrin assay. Compared to the biochemical results of fraction F17, which are quite different, this suggests that the gel filtration column is providing useful separation of the components in the venom. Figure 5 corroborates this interpretation, as it shows the higher fraction numbers becoming increasingly effective at generating apoptosis. All venom fractions and the crude venom itself have shown to be heat labile (Table1) and we have noticed an apparent decrease in activity with storage of the venom at -20°C for 2-3 weeks.

We believe our methods of venom extraction and preparations are well reasoned in that we start with a specific anatomical structure and stimulate a cellular depolarization without physically disrupting the tissue. Our crude venom is then centrifuged and separated by gel filtration chromatography. This size exclusion

chromatographic procedure is similar to other published methods of anemone venom preparations (Honma et al., 2005) (Oliveira et al., 2006) (Oliveira et al., 2004). However, other researchers typically collect only five fractions, where as we collect thirty smaller fractions. Of particular interest to many researchers who collect five fractions, is fraction number three, which has been shown to contain many neurotoxins, in the form of sodium and potassium channel blockers. Zarahenko and collaborators have shown that fraction number three from *B. cangicum* venom, obtained by electro-stimulation, contains at least eighty distinct components. (Zaharenko et al., 2008) We believe our crude venom to be less complex.

The majority of research regarding anemone proteins and peptides has focused on their ion channel regulating capabilities, a few studies have looked into other important physiological processes such as cell death mechanisms. Bartosz and collaborators have shown that acroraghial venom induces tissues necrosis through generation of reactive oxygen species (Bartosz et al., 2008), while the Sanderson group has shown that anemone venom obtained from the milking technique induces apoptosis in human breast cancer cells (Ramezanpour et al., 2013). The same group has also refined the apoptosis mechanism to intrinsic (mitochondrial dependent) pathways of the same venom in lung cancer cells (Ramezanpour et al., 2014).

We know of only one report of anemone venom shown to be from non-nematocyte sources. Moran and collaborators have shown venom to be associated with an

ectodermal gland in close proximity to tentacle nematocyte batteries (Moran et al., 2012). Marymegan Daly published a thorough anatomical study of the vesicles and verrucae (a similar columnar protuberance not found in *Bunodosoma*) in three anemone species including *Bunodosoma californica*. She found no nematocytes associated with vesicles (Daly, 2015). Therefore, the polypeptide venom investigated in this study may be another example of anemone venom that is not housed in nematocytes.

What may be the normal function of this venom? Vesicles could serve in defense of the animal, as the entire column of *B. cangicum* may be covered with over one hundred of these structures (Figure 1). However, *B. cangicum* is usually found buried in the sandy substrate with only its catch tentacles exposed. Could the vesicles provide protection from some subterranean threat? Or perhaps the vesicles defend the animal when it is forced to relocate? These are remaining questions. We also have observed numerous anemones *B. cangicum* attached to large rocks with fully exposed columns, so the animal is not always partially buried in the sand. Finally, it cannot be discounted at this time that perhaps this venom may be a metabolite mixture that serendipitously induces apoptosis in the ZF-L line.

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CONSIDERAÇÕES FINAIS:

Em nossos ensaios percebemos que mesmo após purificar ainda mais a toxina através da centrifugação e filtração em gel, continuamos observando os efeitos apoptóticos, os nossos dados sugerem uma nova metodologia para extração das toxinas da anêmona *Bunodosoma cangicum,* esta metodologia extrai uma toxina mais pura, já que não ocorre mistura de outros subprodutos encontrados ao longo do corpo do animal, também evidenciamos que anêmonas da espécie *Bunodosoma cangicum* possuem potentes toxinas capazes de causar apoptose, estas toxinas são oriundas de vesículas encontradas na coluna da anêmona, até então subprodutos desta região não eram estudados. Para avançar os estudos com estes compostos torna-se necessário realizar uma purificação maior. Em nossa separação através da coluna de Sephadex conseguimos atingir nosso objetivo e verificar frações de toxina que continham efeito e outras que não causavam efeito apoptótico nas células. Através da utilização da técnica de HPLC, podemos caracterizar ainda melhor os efeitos de suas toxinas, verificando quais os componentes daquela amostra e também separando as frações por peso molecular. Futuramente nós iremos caracterizar os compostos presentes na toxina da anêmona *Bunodosoma cangicum* e verificar quais os efeitos que as toxinas extraídas das anêmonas podem causar em outros modelos celulares.

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