Fabio Montoni

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências - Toxinologia do Instituto Butantan para obtenção do título de Mestre em Ciências.

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Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Cópia do formulário existente - ID 4959200217Análise proteômica de tecidos cerebrais de camundongos da linhagem Swiss sob o efeito da peçonha de Crotalus durissus terrificus", protocolada sob o CEUA nº 5593290818 (ID 001400), sob a responsabilidade de **Leo Kei Iwai e Fabio Montoni** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Instituto Butantan (CEUAIB) na reunião de 20/09/2018.

We certify that the proposal "Cópia do formulário existente - ID 4959200217 Proteomic analysis of Swiss mouse brain tissues under the effect of Crotalus durissus terrificus venom", utilizing 95 Heterogenics mice (95 males), protocol number CEUA 5593290818 (ID 001400), under the responsibility of **Leo Kei Iwai and Fabio Montoni** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Butantan Institute (CEUAIB) in the meeting of 09/20/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 09/2018 a 08/2019 Área: Lab. Especial de Toxicologia Aplicada

Origem:	Biotério Central				
Espécie:	Camundongos heterogênicos	sexo: Machos	idade:	40 a 48 dias	N: 95
Linhagem:	Swiss		Peso:	18 a 22 g	

Local do experimento: Biotério do Laboratório Especial de Dor e Sinalização (LEDS)

São Paulo, 22 de julho de 2019

Maria Leonor Sarno de Oliveira
Coordenador da Comissão de Ética no Uso de Animais
Instituto Butantan

Nancy Oguiura
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Instituto Butantan

Dedico esta tese ao meu pai Antonio Montoni Sobrinho e à minha mãe Carmélia Alves Montoni com todo meu amor.

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Este texto apesar de longo, fala apenas sobre uma coisa. A coisa que mais estimo. Amizade.

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"Be all, and you'll be the end all
Life can be a real ball
State of mind
Euphoria".

RESUMO

MONTONI, Fabio. **Análise proteômica de tecidos cerebrais de camundongos da linhagem *Swiss* sob efeito da peçonha de *Crotalus durissus terrificus***. 2019. 82 p. Dissertação (Mestrado em Ciências - Toxinologia) – Instituto Butantan, São Paulo, 2019.

Anualmente, cerca de 2,7 milhões de pessoas sofrem acidentes por serpentes peçonhentas em todo o mundo. Dentre elas, existem relatos de que ocorrem mais de 130.000 mortes e 490.000 amputações e outros problemas graves de saúde causados por este tipo de acidente. No Brasil, dentre as diferentes serpentes distribuídas pelo país, as serpentes do gênero *Crotalus*, mais conhecida como Cascavel é a segunda maior responsável dos acidentes no país ficando atrás apenas das *Bothrops*, conhecidas como jararacas. As peçonhas das *Crotalus* são conhecidamente neurotóxicas e miotóxicas e apesar de existirem uma vasta descrição dos aspectos clínicos e bioquímicos dos efeitos do envenenamento, existem poucos registros descrevendo o envenenamento em um organismo a nível proteômico. Dessa forma, neste estudo avaliamos o efeito da peçonha da serpente *Crotalus durissus terrificus* em cerebelos de camundongos de linhagem *Swiss* após 1, 6, 12 e 24 h depois da injeção da peçonha e avaliamos seus efeitos proteômicos usando espectrometria de massas de alta resolução. Diversas ferramentas de bioinformática foram utilizadas para obter uma visualização geral da variação de algumas das 3600 proteínas identificadas, dos diferentes termos e funções biológicas dos efeitos do envenenamento ao longo do tempo até 24 h. Observamos a redução de termos envolvendo sinalização de sinapses inibitórias, stress oxidativo, manutenção de células neuronais e um aumento em termos envolvendo dano tecidual e fatores ligados a apoptose. Essa análise permitiu revelar a nível molecular, possíveis alvos moleculares específicos da peçonha. Esses dados poderão sugerir novas abordagens terapêuticas (i.e. dirigidas aos alvos proteicos inicialmente afetados pelo envenenamento) para o tratamento do envenenamento pela peçonha da *C. d. terrificus*.

Palavras-chave: Proteoma. Espectrometria de Massas. *Crotalus*. Peçonhas. Envenenamento. Cerebelo. Camundongos.

ABSTRACT

MONTONI, Fabio. **Proteomic analysis of brain tissues of mice of the Swiss strain under the effect of *Crotalus durissus terrificus* venom**. 2019. 82 p. Master's thesis (Master's degree in Sciences - Toxinology) – Instituto Butantan, São Paulo, 2019.

About 2.7 million people are bitten annually by venomous snakes worldwide. Among them, there are more than 130,000 deaths and 490,000 amputations and other serious health problems caused by this type of accident. In Brazil, among the different snakes distributed throughout the country, the snakes of the *Crotalus* genus known popularly as rattlesnakes is the second largest responsible for accidents in the country only behind the *Bothrops* genus of snakes known as jararacas. *Crotalus* snakes venoms are known to be neurotoxic and myotoxic and although there is a wide description of the clinical and biochemical aspects of the effects of *Crotalus* envenoming, there are few works describing the molecular events in an organism due to its envenomation. Thus, in this study we evaluated the effect of *Crotalus durissus terrificus* snake venom on cerebellum of Swiss mice after 1, 6, 12 and 24 h after venom injection and evaluated their proteomic effects using high resolution mass spectrometry. Several bioinformatics tools were used to obtain an overview of the variation of over 3,600 identified proteins, different terms and biological functions of the effects of envenomation over the time up to 24 h. We were able to observe a reduction in terms involving inhibitory synapse signaling, oxidative stress, maintenance of neuronal cells, and an increase in terms involving tissue damage and apoptosis factors. This analysis allowed us to reveal possible molecular targets of the venom. These data may suggest new therapeutic approaches (i.e. directed to protein targets initially affected by the envenomation) for the treatment of envenomation by *C. d. terrificus* venom.

Keywords: Proteome. Mass Spectrometry. *Crotalus durissus terrificus.* Venom. Envenomation. Cerebellum. Mouse.

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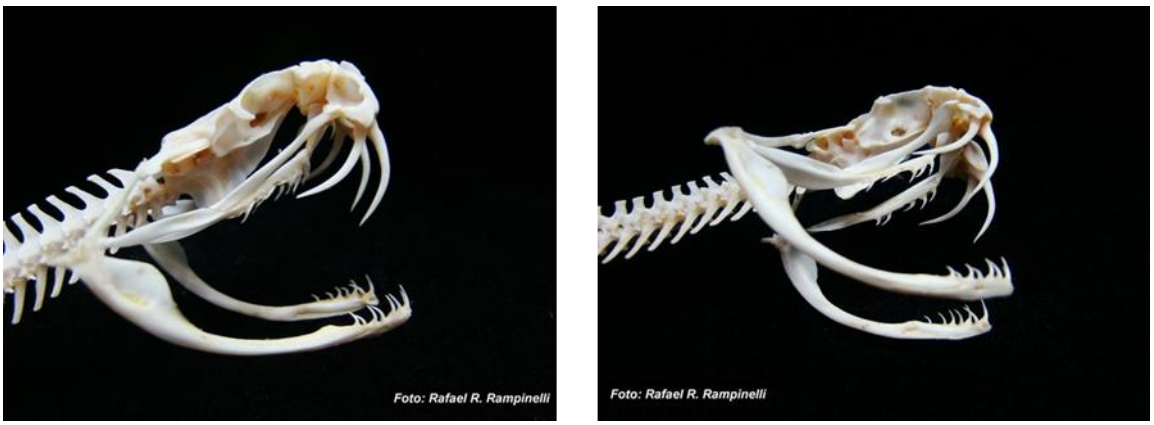
1 INTRODUÇÃO

Segundo a Organização Mundial da Saúde, os acidentes ofídicos são um dos problemas de saúde negligenciados que mais afetam países tropicais e subtropicais. Todo ano, cerca de 5,7 milhões de pessoas sofrem acidentes em todo mundo por serpentes, onde destes, 2,7 milhões dos acidentes são causados por serpentes peçonhentas. Por ano, registra-se mais de 130.000 mortes e 490.000 amputações e outros problemas severos causados por este tipo de acidente (OMS 2017).

Os locais onde mais ocorre este tipo de acidente são na África e Ásia. No continente asiático são dois milhões de acidentes, enquanto no continente africano são aproximadamente 580.000 acidentes. As pessoas mais afetadas por acidentes ofídicos são mulheres, crianças e trabalhadores rurais de regiões menos desenvolvidas economicamente (OMS 2017).

Dentre as famílias de serpentes, as que possuem maior importância clínica são as da família Viperidae, que são as víboras e crotalíneos e que são as maiores causadoras de acidentes em todo mundo. Elas possuem características marcantes como dentição solenóglifa (Figura - 1) onde na parte inferior de suas presas retráteis se encontram as glândulas de peçonha e também possuem um orifício termorreceptor chamado fosseta loreal (Underwood 1967), com exceção da subfamília Viperinae (Ashe, Marx and Inger 1988).

Figura 1 - Crânio da serpente *Crotalus durissus terrificus*.



Fonte: Rafael R. Rampinelli, em trabalho realizado para a Fundação Parque Zoológico de São Paulo.
Um exemplo de dentição solenóglifa entre os membros da família Viperidae

A família Viperidae possui no total 329 espécies em 35 gêneros pertencentes a três grandes subfamílias: Azemiopinae, que contem apenas duas serpentes peçonhentas do gênero *Azemiops*, que se restringem á Ásia, Europa e África (Phelps 2010, Uetz and Hošek 2014); Viperinae, que são as “víboras verdadeiras”, também se restringem á Ásia, Europa e África, possuem 98 espécies descritas (Uetz and Hošek 2014), e Crotalinae, que é a maior subfamília dentre todas e mais diversa, contendo 229 espécies (Uetz and Hošek 2014) e que podem ser encontradas em todo o mundo. Dentre essas três subfamílias, a única que ocorre no continente americano é a das serpentes pertencentes à subfamília Crotalinae, onde 12 gêneros ocorrem no continente: *Agkistrodon*, *Atropoides*, *Bothriechis*, *Bothriopsis*, *Bothrocophias*, *Bothrops*, *Cerrophidion*, *Crotalus*, *Lachesis*, *Ophryacus*, *Porthidium* e *Sistrurus* (Campbell 2004).

Quando se trata de suas peçonhas, as serpentes produzem uma complexa mistura de mais de 100 proteínas (Fox and Serrano 2005) em glândulas especializadas que estão pareadas na parte ventral posterior dos olhos denominada peçonha. Esta faz parte de poucas famílias maiores de proteínas tais como: enzimas (serino peptidades, metaloproteinases, L-amino ácido oxidase, PLA2) e até mesmo proteínas sem atividade enzimática (Ohanina, desintegrinas, lectinas de tipo C, miotoxinas) que conferem à peçonha características distintas como, por exemplo, hemotoxicidade, cardiotoxicidade, citotoxicidade e até mesmo neurotoxicidade (Jin and Varner 2004, White 2005). Neste último caso, ao atingir a presa, a peçonha afeta o sistema hemostático e reparo tecidual, causando distúrbios na coagulação, hipofibrinogenia e pode também causar necrose tecidual local (Markland 1998, Fox and Serrano 2005). Estas ações podem ser resultados da ação de moléculas isoladas ou uma ação sinérgica de proteínas, carboidratos, lipídeos, peptídeos, enzimas e outras moléculas pequenas (Fox and Serrano 2005, Tanjoni et al. 2005, Calvete, Juarez and Sanz 2007, Doley and Kini 2009, Gutiérrez et al. 2010).

Analisando evolutivamente, pode-se concluir que toxinas provêm a partir do período da radiação dos Colubridae, onde proteínas que desempenhavam papéis fisiológicos normais foram ao longo do tempo recrutadas para fazer parte de um estrito proteoma (Fry et al. 2006, Fry and Wüster 2004), resultado de uma evolução Darwiniana positiva acelerada que permitiu que as serpentes pudessem transitar do uso da constrição para a inoculação de uma substância química, que permite o uso

desta defesa ativa como forma de dominação, paralisação, morte e digestão de presas até mesmo muito maiores que a serpente (Lynch 2007).

No Brasil, segundo o Ministério da Saúde, estima-se que em 2018 ocorreram mais de 22.800 acidentes com serpentes peçonhentas, sendo estes causados pelos quatro gêneros de serpentes peçonhentas com várias subespécies descritas existentes em nosso território nacional: *Bothrops*, conhecidas como as jararacas, responsáveis por 90,5% dos acidentes; *Micrurus* (que não são pertencentes á família Viperidae, mas sim Elapidae), conhecidas como corais, responsáveis por 0,4% dos acidentes; *Lachesis*, conhecidas como surucucus, responsáveis por 1,4% dos acidentes e por fim, as *Crotalus*, conhecidas como cascavéis, responsáveis por 7,7% dos acidentes. Com relação à sua letalidade, a média entre os gêneros é de 0,45%, com exceção do gênero *Crotalus* que chega aos 1,87% (Amaral et al. 1986). Com relação à sua distribuição, com exceção das serpentes do gênero *Lachesis*, que podem ser encontradas apenas na região amazônica e as serpentes do gênero *Crotalus*, que se distribuem preferencialmente na região sul e sudeste, os outros gêneros podem ser encontrados em todo o território nacional (Azevedo-Marques, Hering and Cupo 2003b).

Abordando mais especificamente a serpente *Crotalus durissus* (Linnaeus, 1758) (Figura - 2), ela é uma serpente terrícola de hábitos predominantemente noturnos e possui uma dieta que pode incluir mamíferos e até mesmo aves (Vanzolini, Ramos-Costa and Vitt 1980). Esta serpente habita predominantemente vegetações do cerrado e caatinga, porém, também possuem uma alta adaptabilidade e podem ser vistas em áreas que foram antropizadas. Sua característica mais marcante é a presença de um “guizo” no fim de sua cauda, que na verdade, é uma estrutura de quitinina que se especula ser usada como “aviso” caso se sinta acuada (Campbell and Lamar 1989).

Figura 2 - *Crotalus durissus terrificus*

Fonte: Observatório de Justiça & Conservação
Imagem da serpente *Crotalus durissus terrificus*.

Sua peçonha contém neurotoxinas de ação pré-sináptica que atuam nas terminações nervosas inibindo acetilcolina causando paralisias motoras. Dentro das primeiras seis horas de inoculação são relatados pelos pacientes diversos sintomas relacionados à visão, tais como, ptose palpebral uni ou bilateral, oftalmoplegia, visão turva, diplopia, sendo esta uma consequência derivada da paralisia da musculatura extrínseca e intrínseca do globo ocular, após o comprometimento do terceiro par dos nervos cranianos (Azevedo-Marques, Cupo and Hering 2003a).

A peçonha de serpentes do gênero *Crotalus* possuem cinco componentes majoritários, sendo estes: crotoxina, crotamina, (Gonçalves 1956) giroxina (Barrio 1961, Barrabin et al. 1978), convulxina (Prado-Franceschi and Brazil 1981) e crotapotina (Fortes-Dias et al. 1994):

- **Crotoxina**

É a neurotoxina mais abundante (65% do peso seco), na peçonha desta serpente. Em 1938, Slota e Fraenkel-Conrat realizaram experimentos onde a

crotoxina foi isolada e posteriormente cristalizada (Slotta and Fraenkel-Conrat 1938). Após diversos estudos, foi concluído que esta subunidade da peçonha possuía uma conformação bem definida e peso molecular de 30,000 Da (Slotta and Fraenkel-Conrat 1938). A peçonha possui ação neurotóxica e uma ação hemolítica secundária (Brazil 1966). Em 1955 descobriu-se que estas ações eram atribuídas por duas subunidades (Neumann et al. 2010):

- ✓ **Fosfolipase A2:** Inibe a liberação de neurotransmissores na junção neuromuscular e também possui ação miotóxica. Apenas se encontra ativa quando está associada a crotapotina (Fortes-Dias et al. 1994).
- ✓ **Crotapotina:** Subunidade ácida da crotoxina. Ela inibe a atividade enzimática e possui a capacidade de potencializar a fosfolipase A2 (Fortes-Dias et al. 1994, Fernandes et al. 2017).

Além de seu papel principal na neurotoxicidade (Brazil 1966), a crotoxina também pode apresentar nefrotoxicidade (Hadler and Brazil 1966), miotoxicidade (Breithaupt 1976) e cardiotoxicidade (Fortes-Dias et al. 1994, Fernandes et al. 2017).

- **Crotamina:** Foi isolada primeiramente em 1956 (Gonçalves 1956). Estudos apontam que sua função biológica e papel bioquímico está diretamente relacionado com os efeitos miotóxicos (Bieber and Nedelkov 1997). Em 1980 sua estrutura foi purificada e sua estrutura de nucleotídeos foi elucidada (Rádis-Baptista et al. 1999). Alguns outros estudos apontam que a crotamina tem a capacidade de penetrar a membrana celular e pode ser utilizada como marcadores de células vivas sem alterar sua função biológica (Kerkis et al. 2004).
- **Giroxina:** Ela foi descrita nos anos 60 (Barrio 1961), mas só chegou a ser isolada em 1978 (Barrabin et al. 1978). Esta toxina é muito famosa por causar grave perda de equilíbrio e constante giro sobre o próprio eixo em camundongos que foram submetidos á injeção da toxina. Em casos mais graves, esta toxina pode também causar fortes contrações involuntárias constantes nos músculos (Kozako et al. 2002).
- **Convulxina:** Foi isolada pela primeira vez por Júlia Prado Franceschi (1981) (Prado-Franceschi and Brazil 1981). Neste estudo revelou-se que a convulxina conserva os mesmos efeitos da giroxina em testes feitos em camundongos. No mesmo estudo, foi relatado que aplicação intravenosa em cachorros anestesiados causaram uma intensa estimulação respiratória seguida de apneia, seguida de forte queda e posterior forte hipertensão transiente. (Prado-Franceschi and Brazil 1981).

Além de sua atividade neurotóxica, a peçonha da cascavel pode apresentar atividade miotóxica e atividade coagulante, sendo a primeira causadora de dolorosas lesões sistêmicas contra as fibras musculares que leva a uma liberação de enzimas e mioglobinas para o sangue, que é excretado pela urina com coloração marrom-avermelhado. A segunda é derivada de uma fração que contém a enzima tipo trombina, que causa distúrbios na coagulação sanguínea em torno de 40% dos pacientes (Azevedo-Marques et al. 2003a) e em alguns raros casos (que podem ocorrer com mais frequência do que se possui registro), o paciente pode apresentar uma sensação de corpo estranho na garganta, dor que pode persistir diversas semanas após o acidente. Além disso, ocorrem alterações no sistema nervoso central (Jorge and Ribeiro 1992) que podem indicar que a peçonha da cascavel causa muitos efeitos que podem ser importantes no contexto do acidente crotálico e que não foram ainda elucidados. Todas as ações aqui descritas demonstram a gravidade de um acidente ofídico, em especial um acidente crotálico e também, a necessidade de métodos para a rápida neutralização destas toxinas do organismo do indivíduo acidentado.

Apesar da severidade e grande dispersão do problema ao longo de diversas regiões do mundo, o acidente ofídico pode ser tratado de forma eficaz através do soro antiofídico descoberto por Albert Calmette (Calmette 1896) no ano de 1859. Este estudo abriu as portas para que Vital Brazil em 1901 criasse o primeiro estudo epidemiológico (chamado de *Boletim para Observação de Accidente Ophidico*), juntamente com a distribuição das primeiras ampolas de soro antiofídico desenvolvidas da forma como conhecemos atualmente (Bochner and Struchiner 2003) e que foi sendo aprimorado ao longo do tempo e sendo utilizados em diversos sistemas de saúde em todo mundo. A partir daí, o uso deste tratamento se tornou universal, porém, muitos países especialmente na Ásia e África tem baixa demanda de empresas que produzem o antipeçonha encarecendo o produto. Além disso, as poucas empresas que o produzem carecem de soros efetivos, tornando assim um agravante para o tratamento deste tipo de emergência (OMS 2017).

Diversos estudos abordam o quadro do acidente ofídico no aspecto clínico e bioquímico (Jorge and Ribeiro 1992) e muitos outros estudam a composição das peçonhas destas serpentes a nível genômico, transcriptômico e proteômico, ou até mesmo avaliam os efeitos de moléculas específicas desta avaliando seus efeitos de

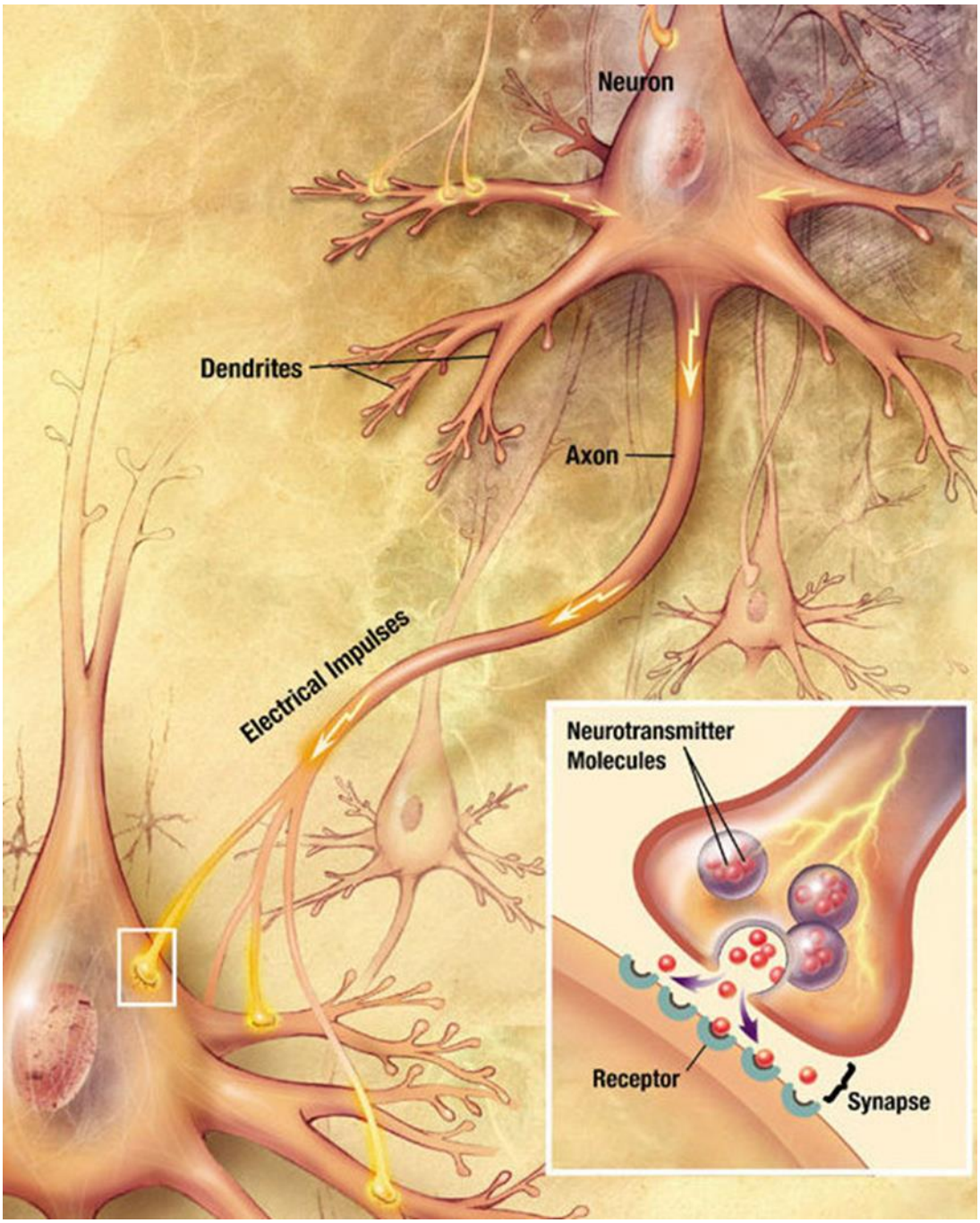
forma isolada, porém, até o momento nenhum grupo de pesquisa realizou um estudo do efeito da peçonha total a nível molecular sob algum tecido específico.

Por conta da ação neurotóxica da peçonha de *Crotalus* e o relato de Jorge e Ribeiro no ano de 1992 (Jorge and Ribeiro 1992) onde se apontam casos de danos no sistema nervoso central, acreditamos que o encéfalo seja uma região importante afetada em um acidente crotálico.

O encéfalo é a principal estrutura do sistema nervoso central de vertebrados e de alguns invertebrados. É responsável por determinar respostas de diversos tipos de estímulos, onde estes envolvem diversos sistemas fisiológicos e comportamentais para gerar uma resposta do organismo (McEwen 2007). Um encéfalo humano possui cerca de 100 bilhões de neurônios que formam trilhões de conexões entre eles. Os neurônios são as células responsáveis pela sinalização do sistema nervoso. São células alongadas, constituintes de um axônio, que é a estrutura responsável por transmitir os impulsos elétricos. Através deste impulso elétrico, ao chegar nas sinapses, acontece a neurotransmissão química, que será enviada até o neurônio seguinte formando assim, uma resposta a determinado estímulo.

Dentre as diferentes estruturas do encéfalo, o cerebelo é o responsável pela coordenação, aprendizagem motora e equilíbrio. Acreditamos que, como mostrado por Silva e colaboradores em 2011, alguns componentes da peçonha tais como a giroxina pode aumentar a permeabilidade da barreira hematoencefálica permitindo assim, uma passagem de componentes por ela causando efeitos diretos em determinadas regiões do encéfalo e não apenas um efeito sistêmico causado pela peçonha (da Silva, Oliveira and Camillo 2011).

Figura 3 - Exemplo de um neurônio e suas estruturas básicas.



Fonte: Imagem criada pelo NIA (*US National Institutes of Health, National Institute on Aging*).
Exemplo do funcionamento de um neurônio. O impulso elétrico gerado no neurônio percorre o axônio, que, ao atingir a sinapse, gera a liberação de neurotransmissores químicos que por fim irão chegar em outras células gerando uma resposta.

A análise proteômica baseada em espectrometria de massas avançou muito nos últimos 15 anos com o desenvolvimento de equipamentos mais sensíveis que possibilitam analisar proteomas complexos que permitem identificar e quantificar

milhares de proteínas em diversas amostras biológicas. No contexto toxinológico, esta tecnologia tem sido utilizada majoritariamente para a caracterização proteica de peçonhas de diferentes espécies de serpentes visando objetivos distintos tais como estudos fenotípicos (Gonçalves-Machado et al. 2016, Tang et al. 2017, Nicolau et al. 2017), estudos sobre a evolução das espécies (Sanz and Calvete 2016, Junqueira-de-Azevedo et al. 2016), e estudos caracterizando variações interespécies (Gonçalves-Machado et al. 2016, Reeks et al. 2016). Sendo assim, nosso grupo de pesquisa considera oportuna a realização de um estudo do efeito da peçonha da cascavel em cerebelo de camundongo através de uma abordagem proteômica, com a finalidade de revelar o efeito do envenenamento nesta região cerebral á nível molecular.

2 OBJETIVOS

O objetivo deste estudo foi avaliar o efeito proteômico da peçonha da cascavel no cerebelo de camundongos da linhagem *Swiss* e avaliar seus efeitos a nível molecular através de espectrometria de massas de alta resolução. Este estudo pretende elucidar efeitos moleculares ainda não descritos na literatura que possam ser de interesse na compreensão da forma de ação da peçonha no contexto do envenenamento, trazendo assim uma nova abordagem para o estudo dos acidentes ofídicos.

3 ARTIGO SUBMETIDO PARA PUBLICAÇÃO

Rattle snake venom impacts on mice cerebellum proteomics points to synaptic inhibition and tissue damage

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Running Title: Snake venom effect on mouse cerebellum proteome

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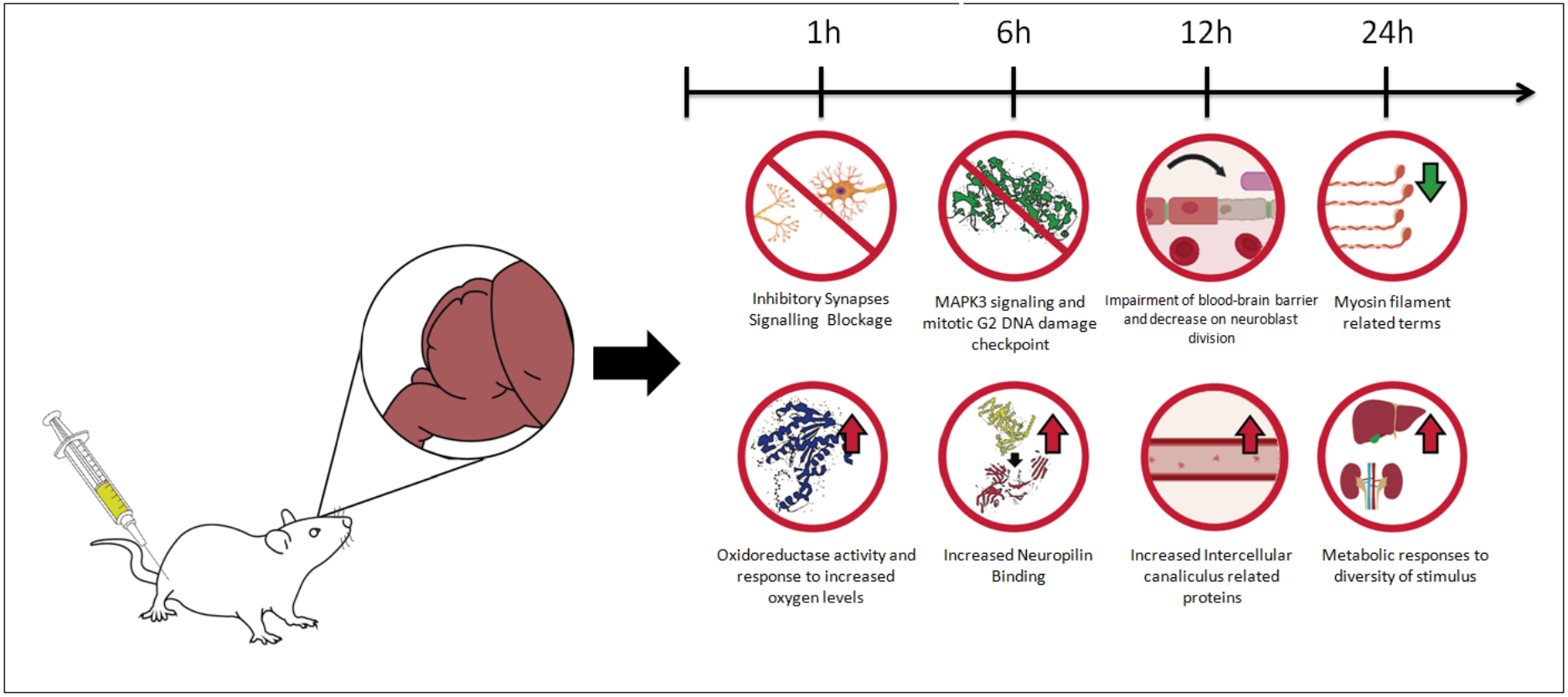
Graphical Abstract



Highlights

- Mice injected with *C.d.terrificus* venom show several protein abundancy changes in cerebellum

- Several biochemical processes changes over the time with venom such as metabolic process and tissue damage

- Each time point analyzed showed increase or decrease of terms such as oxidative stress, DNA repair and synapses

- Network analysis show diverse proteins disturbances that might be directly related to *Crotalus* envenomation.

Abstract

Snake envenomation is responsible for more than 130,000 deaths and 490,000 amputations worldwide. In Brazil, the *Crotalus* genus known as Rattlesnake is the second largest responsible for snake bite accidents in the country. Although there is a wide description of clinical and biochemical aspects of the effects of *Crotalus* envenoming, there are few works describing the molecular events in an organism due to its envenomation. In this study we evaluated the effect of *Crotalus durissus terrificus* snake venom on cerebellum of mice after 1, 6, 12 and 24h after venom injection and evaluated their proteomic effects using mass spectrometry. The analysis of the variation of over 3,600 identified proteins along with the time showed a reduction in terms involving inhibitory synapse signaling, oxidative stress, maintenance of neuronal cells, and an increase in terms involving tissue damage and apoptosis factors. This analysis allowed us to reveal possible molecular targets of the venom showing new aspects of snake envenomation effect. These data may contribute for new therapeutic approaches (i.e. directed to protein targets affected by the envenomation) for the treatment of envenomation by *C. d. terrificus* venom.

Keywords: Mass Spectrometry, proteome analysis, *Crotalus durissus terrificus*, snake venom, *Mus musculus*, cerebellum, envenomation

Significance Statement

Snakebite is a neglected global health problem that affects mostly rural and tropical areas of developing countries. It is estimated that over 5.4 million people are bitten by snakes each year, from which 2.7 million people are bitten by venomous snakes resulting in disabilities such as amputations and in some cases leading to death.
The *Crotalus durissus terrificus* snake is the most lethal snake in Brazil and the study of the molecular aspects of envenomation in a specific tissue in a proteomic level may reveal novel aspects of the envenomation that may reveal new forms to look at the envenoming by *C. d. terrificus* snake to better understanding the envenomation process and to help in the development of new anti-venom additives.

1. Introduction

According to the World Health Organization, snakebites are one of the neglected health problems that affect mostly tropical and subtropical countries. Every year, venomous snakes have been causing ~2.7 million accidents worldwide resulting in 130,000 deaths, 490,000 amputations, and other severe health problems [1]. In Brazil, according to the Brazilian Ministry of Health, 22,000 accidents occur with venomous snakes every year [2]. These accidents are mainly caused by four main genera of venomous serpents found in the Brazilian territory: *Bothrops*, responsible for 90.5% of the accidents; *Micrurus*, responsible for 0.4% of accidents; *Lachesis*, responsible for 1.4% of accidents, and *Crotalus*, known as rattlesnakes, are responsible for 7.7% of accidents. Although Crotalus accidents is the second largest responsible for snakebite accidents in the country, its lethality is the highest reaching 1.87% [3].
Crotalus durissus snakes venom is mainly composed of a mixture of peptides and other minor chemical compounds, among which it contains presynaptic neurotoxins that act on nerve endings inhibiting acetylcholine, causing motor paralysis [4, 5]. Within the first six hours of inoculation, patients report several eye-related symptoms, such as palpebral, uni- or bilateral ptosis, ophthalmoplegia, blurred vision and diplopia which is a consequence of the paralysis of the extrinsic and intrinsic musculature of the eyeball after impairment of the third pair of cranial nerves [6]. In addition to its neurotoxic activity, myotoxic and abnormal coagulant activity of the

venom have been observed. The myotoxic activity can be observed in most serious cases where various painful systemic lesions against the muscular fibers occurs, which leads to a release of enzymes and myoglobin to the blood that is excreted by the urine that turns to a reddish-brown color due to them. The abnormal coagulant activity comes from a fraction containing the thrombin-like enzyme, which causes disturbances in blood coagulation in about 40% of patients [7]. Besides its role in neurotoxicity [8], *C. durissus* venom have also been described to be nephrotoxic [9], myotoxic [10] and cardiotoxic [11, 12]. *C. durissus* venom composition analysis have revealed four major components: crotoxin, that is the most abundant toxin in the venom (65% of dry weight) with a neurotoxic action and a secondary hemolytic action [13]; crotamine, that has been related to the myotoxic effects [14]; gyroxin, that causes strong involuntary contractions of the muscles and severe loss of balance making the mice to spin on its own axis after subjecting the animals to toxin injection [15]. In addition, gyroxin has also been described to transpose the blood-brain barrier [16]; and convulxin, that has been described to have the same effects of gyroxin in tests performed in mice in addition to an abrupt and transient fall of arterial blood pressure and late hypotension in dogs [17].

These descriptions reveal the venom action severity of the crotalic accident and the need for methods for a rapid neutralization of these toxins in an injured individual. Nowadays the antivenom antiophidic sera, first described by Albert Calmette in 1859 [18] has been still used to treat the snakebite. These sera are developed by injecting small amounts of snake venom in horses and the antibodies formed against this venom is purified. However, many countries especially in Asia and Africa have a low demand of companies that produce the antiophidic sera making it an aggravating problem for the treatment of this type of medical emergency in these areas [1].

Several studies have shown the clinical features of snakebite and many others have studied the venom composition of these snakes at genomic, transcriptomic and at the proteomic level, and also evaluated the effects of specific molecules from Crotalus snake venom thus evaluating their effects in isolation. However, no research group has yet conducted a study of the effect of *C.d.terrificus* venom at the molecular proteomic level at any specific tissue.

In order to obtain a comprehensive proteomic characterization of the effects of the *Crotalus durissus terrificus* venom in the cerebellum of mice, responsible for equilibrium, coordination, balance, motor learning, motor memory, and motor

consolidation, we used a high resolution mass spectrometry-based proteomics to analyze cerebellums of mice injected with the venom at different time points, that comprises the period when patients seeks the hospital after snakebite [19].

2. Experimental Section

2.1. *Crotalus durissus terrificus* venom

Venom was extracted in the herpetology department of the Butantan Institute and supplied lyophilized by the Butantan Institute Venom Center (NEVAS). The department pooled 256 snake venom extractions from snakes collected at the state of São Paulo, Goiás, Minas Gerais, Mato Grosso do Sul, and Paraná. The venom pool was quantified according to the curve protocol described by Markwell [20], analyzed by SDS-PAGE according to protocol described by Laemmli in 1970 and its median lethal dose (LD50) was determined according to the protocol described by Villarroel [21]. For this lot, the LD50 was established as 0.71 µg / adult mouse weighing between 18 and 22 g.

2.2 Mice

Male mice of SWR / J Swiss strain weighing between 18 and 22 g were bred in polycarbonate boxes at room temperature at 22 ºC, constant humidity, positive pressure and light/ dark cycle of 12 hours and free access to food and water. All mice used in this project were approved by The Ethic Committee on Animal Use of the Butantan Institute under the certification CEUAIB: 4959200217. The methodology applied in this study followed the norms of the National Council for Control of Animal Experimentation (CONCEA) and all associated legislation.

2.3 Treatment of mice with *Crotalus durissus terrificus* snake venom

The venom treatment was performed by injecting 0.5 LD50 (0,36 µg / animal) of the rattlesnake venom solubilized in 0.9% NaCl into the gastrocnemius muscle in a volume of 50 µl with Ultrafine-II BD micro syringe. As a control of the experiment, only the vehicle (0.9% NaCl) was injected at the same time points, volume and the

same animal numbers as the treated mice.

2.4 Tissue acquisition

One hour, 6 h, 12 h and 24 h after the venom injection, mice were anesthetized by inhalation of isoflurane in a small glass chamber. Upon losing consciousness, the animal was removed and immediately sacrificed by decapitation.

For mass spectrometry analysis (n=5 for each condition), the cerebellum was carefully removed with the aid of an appropriate tweezers and scalpels and immediately washed in ice-cold PBS and flash frozen in liquid nitrogen. After all the extractions, samples were kept in -80 ºC freezer.

For the microscopy analysis using HE-staining assay (n=3 for each condition), brains were removed and fixed in 4% PFA (pH 6.9, Merck) for 24 h and stored in 70% ethanol until the paraffin blocks were assembled and cut in 15 µm thickness for further histology analysis.

2.5 Cerebellar tissue protein extraction for mass spectrometry analysis

After tissue dissection, cerebellums were weighed and lysed with PTS lysis buffer composed of 100 mM Tris-HCl pH 9.0, 12 mM SDC (Sodium dodecyl sulfate), 12 mM SLS (Sodium lauryl sulfate), supplemented with Halt protease and phosphatase inhibitors composed of Aprotinin 80 µM, Bestatin 5 mM, E-64 1.5 mM, Leupeptin 2 mM, Sodium Fluoride, Sodium Orthovanadate, Pyrophosphate, β-Glycerophosphate (Halt, Thermo Fisher Scientific). Lysis buffer were added at 8x the volume to the weight of the structure. Immediately after, the cerebellums were homogenized using the Precellys 24 tissue homogenizer (Bertin Instruments, France) at 6800 g for 30 seconds. Samples were then heated at 95 ºC for 5 minutes and sonicated on ice for 20 minutes followed by centrifugation at 14.000 g at 4 ºC for 30 min. The supernatant was collected, and samples were submitted to precipitation of proteins.

Protein precipitation was performed by methanol/ chloroform precipitation which precipitate proteins and also delipidate the sample. Methanol: water: chloroform at 4:3:1 (v:v:v) was added at 1:1 with protein volume followed by centrifugation at

10.000 g for 5 minutes. The aqueous methanol phase was carefully removed, and three volumes of methanol were added, vortexed for 10 seconds and centrifuged at 10.000 g for 15 minutes. The supernatant was carefully removed, and samples were resuspended in 1 mL 0.1 M NaOH. After these procedures, samples were quantified using the BCA Protein Assay Kit (Thermo Fischer Scientific) following the manufacturer's recommendations. In addition, fifteen micrograms of the total extract after precipitation was analyzed in 12% SDS PAGE and stained with colloidal coomassie to verify the consistency of the extracts obtained (Supplemental Figure 1)

2.6 Sample preparation for mass spectrometry analysis

Proteins were digested with trypsin using a modified FASP protocol described by Wiśniewski [22]. Briefly, 100 µg of the total extract were reduced with DTT 0.02 mM and cystein alkylated with iodoacetamide 0.05 mM in the Microcon filter YM-10 MWCO 10 KDa (Millipore) and digested with trypsin (Sigma-Aldrich) in a ratio of 1: 50 (enzyme: substrate). The pH of each sample was checked and if necessary adjusted to pH 8.0 with HCl 0.1 M or NaOH 0.1 M. Digestion was carried out by incubating at 37 ºC for 18 hours. As quality control for digestion, three µg of each digested sample was applied in a 12% SDS PAGE and stained with silver nitrate and compared to the non-digested sample (Supplemental Figure 2).

2.7 Stage-Tip dessalting

Dessalting was performed following the method described by Rappsilber [23] with slight modifications. Briefly, 30 µg of tryptic peptides were dessalted in an in house-made stage tips mounted in a 200 µl pipette tips with three layers of SDB-XC membrane (Empore styrene divinylbenzene extraction disk cartridge, 3M). Membranes were first equilibrated with Methanol 100%, washed with Solution A (5% Acetonitrile, 0.1% TFA), samples were loaded and subsequently washed with Solution A. Samples were eluted with 100 µl of Solution B composed with 80% Acetonitrile, 0.1% TFA. Washes and elution were performed by centrifuging the tips mounted on an Eppendorf tubes at 1000 g for 5 min. After elution, samples were dried in speedvac and resuspended with 0.1% TFA. Samples were quantified using

the Pierce BCA Peptide Assay Kit (Thermo Scientific), following the manufacturer's recommendations using standard protocol.

2.8 Dimethyl labeling of tryptic peptides

Labeling was performed by incubating the protein extract for 2 hours with 20 µL of the light label mix for the control condition (500 µL TEAB 50 mM, 2,8µL de CH_2O 37% and 25 µL of $NaBH_3CN$ 0,6 M) and 20 µL of heavy label mix for the venom treated samples (500 µL of TEAB 50 mM, 5 µL de $13CD_2O$ 20%, 25 µL de $NaBD_3CN$ 0,6 M) for 2 hours at room temperature. The reaction was stopped with 5µL 1 % ammonia and incubated for 30 min at 35 ºC. The control and venom treated extracts were combined at 1:1 proportion for further mass spectrometry analysis. All chemicals were purchased from Sigma-Aldrich.

2.9 Mass spectrometry-based proteomics

Mass spectrometry analysis were performed on a hybrid quadrupole orbitrap Q-Exactive HF (Thermo Scientific, Bremen, Germany) mass spectrometer coupled to an UltiMate 3000 capillary nano LC system (Thermo Scientific, Bremen, Germany) using the shotgun/ bottom up approach in positive ion mode. One microgram of peptides was injected with a gradient of 5 to 50% solvent B (acetonitrile 90%, 0.1% TFA) in 120 minutes at a flow of 200 nL / min. The electrospray source was operated at 2.2 kV. The peptide mixture was analyzed by the acquisition of full scan mode spectra at a resolution of 120.000 for the determination of MS1 with a maximum injection time of 60 ms in the range of 375 to 1500 m / z. Data dependent acquisition of the 20 most intense peaks were automatically selected for the subsequent acquisition of spectra of the product ions to the MS / MS at a resolution of 15.000 with a maximum injection time of 40 ms in a range of 200 to 2000 m / z with a dynamic exclusion of 15 seconds.

2.10 Protein identification

Protein identification of the mass spectrometry data were performed using Peaks Studio [24] version X (Bioinformatics Solutions Inc., Waterloo, ON, Canada) using de

novo sequencing tools for peptide identification and Search DB for the classical search of sequences against the *Mus musculus* database, downloaded from UniProtKB / Swiss-Prot [25] in March 2019. To avoid the presence of contaminants, GPM's cRap bank (The Global Proteome Machine [26]) was used as contaminant database. The parameters used in the software to perform searches for modifications were: carbamidomethylation of cysteine was set as fixed modification and oxidation of methionine and N-terminal acetylation was set as variable modifications. Mass error tolerance for MS and MS/ MS was set to 10 ppm and 0.02 Da, respectively. Trypsin was selected as the proteolytic enzyme used in the digestion of proteins and up to three miss cleavages were allowed. Once the DB search was finished, the FDR (Benjamini- Hochberg) was adjusted to 0.1% and ALC (average local confidence) was adjusted to 80 %.

2.11 Dimethyl Quantification

The quantification of the proteins in the samples was performed by dimethylation method that compares the difference of relative abundance of the ions [27] generated in the MS spectrum. Maxquant software algorithms were used to identify the groups of proteins, to normalize ion spectra intensities and to determine the significance of protein groups [28]. The global parameters for the search were: light labeling for the control samples (Dimethyl Lys0, + 28 Da) and heavy labeling for the venom treated samples (Dimethyl Lys8, + 36 Da). The proteolytic enzyme of choice was set to trypsin with three permitted miss cleavages. Carbamidomethylation of cysteine was chosen as a fixed modification, and oxidation of methionine and acetylation of the N-terminal was set as a variable modification, with a maximum of 5 modifications per peptide allowed. Regarding the instrument parameters, the default settings for Orbitrap was used, with a tolerance of 20 ppm for the first peptide search and 4.5 ppm for the second. The database used in this search was Swissprot *Mus musculus* used in the identification quoted above.

2.12 Hematoxylin Eosin Assay

The slides were stained for H&E with standard procedures [29]. Slides were analyzed using objectives of 5x, 10x, 40x magnification and pixel image of 1.12µm and 0.28µm

respectively. Images were obtained with the TCS SP5 (Leica, Wetzlar, Germany) microscope using × 10/1.25 oil. Blind assessment of all slides images was performed.

2.13 Bioinformatic analysis

2.13.1 Non ranked enrichment analysis

Proteins identified exclusively in venom treated mice over the controls were analyzed using WebGestalt [30] (WEB-based Gene SeT AnaLysis Toolkit) for functional enrichment analysis (webgestalt.org). The overrepresentation enrichment Analysis (ORA) were created for GO biological process, cellular component, and molecular function. The FDR was adjusted for 0.05 using the Benjamini-Rochberg procedure which was used for the statistical test to determine the enrichment. The enrichment was compared between all-time points.

2.13.2 Quantitative Proteomics Analysis

Heatmaps were developed to comprise the protein abundance changes during the envenomation. The data was plotted on R studio and replicates were normalized with log (2) and Z-scored to provide a visualization of the total protein change profile. For the individual protein heatmap plot, samples were normalized with the log (2) and each replicate were submitted to Welch T-test and significant proteins were plotted using Perseus tools.

The fold change and the Protein-Protein interaction provided by String database [31] information were uploaded into Cytoscape [32] open source platform (cytoscape.org) to create a network and visualize the change profile of protein quantities during the envenomation time points. The Auto Annotate tool [33] was used to cluster the protein network into GO [34] molecular function.

3. Results

In order to obtain a comprehensive proteomic molecular characterization of the effects of the *Crotalus durissus terrificus* venom in the cerebellum of mice, we used a

high resolution mass spectrometry-based proteomics to analyze cerebellums of mice injected with the *C. d. terrificus* venom at different time points, that comprises the period when patients seeks the hospital after snakebite [19]. Thirty-two mice were injected with *C. d. terrificus* venom at the gastrocnemius muscle and 32 animals were injected with saline control. Five mice from each group were sacrificed at 1h, 6 h, 12 h and 24 h post venom or saline injection for mass spectrometry–based proteomics analysis and three mice from each group were sacrificed at these same time points for brain histology analysis.

3.1 Total extract of cerebellum protein acquisition

Cerebellum tissue preparation for mass spectrometry analysis yielded 340 to 520 µg of sample (table 1, supplemental table S1). Each sample were lysed with PTS buffer, reduced with DTT, alkylated with IAA and digested with trypsin. Digested samples were isotopically labeled with light and heavy formaldehyde and desalted using an in-house prepared stage tips. Samples were analyzed using a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer and the generated data were analyzed on Peaks X and Maxquant softwares for identification and quantification of proteins respectively. The obtained data set were further analyzed as their biological function and displayed in forms of comparative charts, graphs, heatmaps and network views. All .raw data were uploaded and stored at the Center for Computational Mass Spectrometry of the University of California, San Diego, MassIVE website and can be downloaded from ftp://MSV000084135@massive.ucsd.edu/Montoni_F_et.al
(user login: leoiwai, password: Proteoma13)

Table 1 – Quantity of total protein extract obtained from each mouse / mg.

	Replicate #					
Sample	**1**	**2**	**3**	**4**	**5**	**Mean**
1h CTRL	0.45	0.52	0.52	0.45	0.51	0.49
1h CDTv	0.44	0.39	0.45	0.49	0.61	0.484
6h CTRL	0.47	0.51	0.48	0.57	0.45	0.5
6h CDTv	0.47	0.48	0.45	0.47	0.23	0.42
12h CTRL	0.45	0.38	0.34	0.38	0.36	0.38
12h CDTv	0.42	0.33	0.35	0.46	0.38	0.39
24h CTRL	0.39	0.42	0.34	0.38	0.42	0.39
24h CDTv	0.39	0.42	0.41	0.41	0.4	0.41

CTRL = control CDTv = *Crotalus durissus terrificus* venom

Note: The supplemental figures 1-4 and tables 1-4 and all raw data are provided at the Center for Computational Mass Spectrometry of the University of California, San Diego, MassIVE website and can be downloaded from ftp://MSV000084135@massive.ucsd.edu/Montoni_F_et.al/, login: leoiwai, password: Proteoma13

3.2 Overview of identified proteins

A total of 4614 protein groups were identified (Supplementary table 1), with a FDR score of 0.1% for peptides and 11.3% for proteins, The analysis of the five replicates of each condition showed a high overlap of >80% of identified proteins (Supplemental Figure 3).

Among the 4614 proteins identified, 1938 proteins were identified in all time points at both control and venom treated samples (Supplemental Figure 3A). The global profile of all identified proteins was listed and compared (Figure 1). The comparison among exclusiveness of identification in venom treated mice compared to controls at different time points is shown in figure 2.

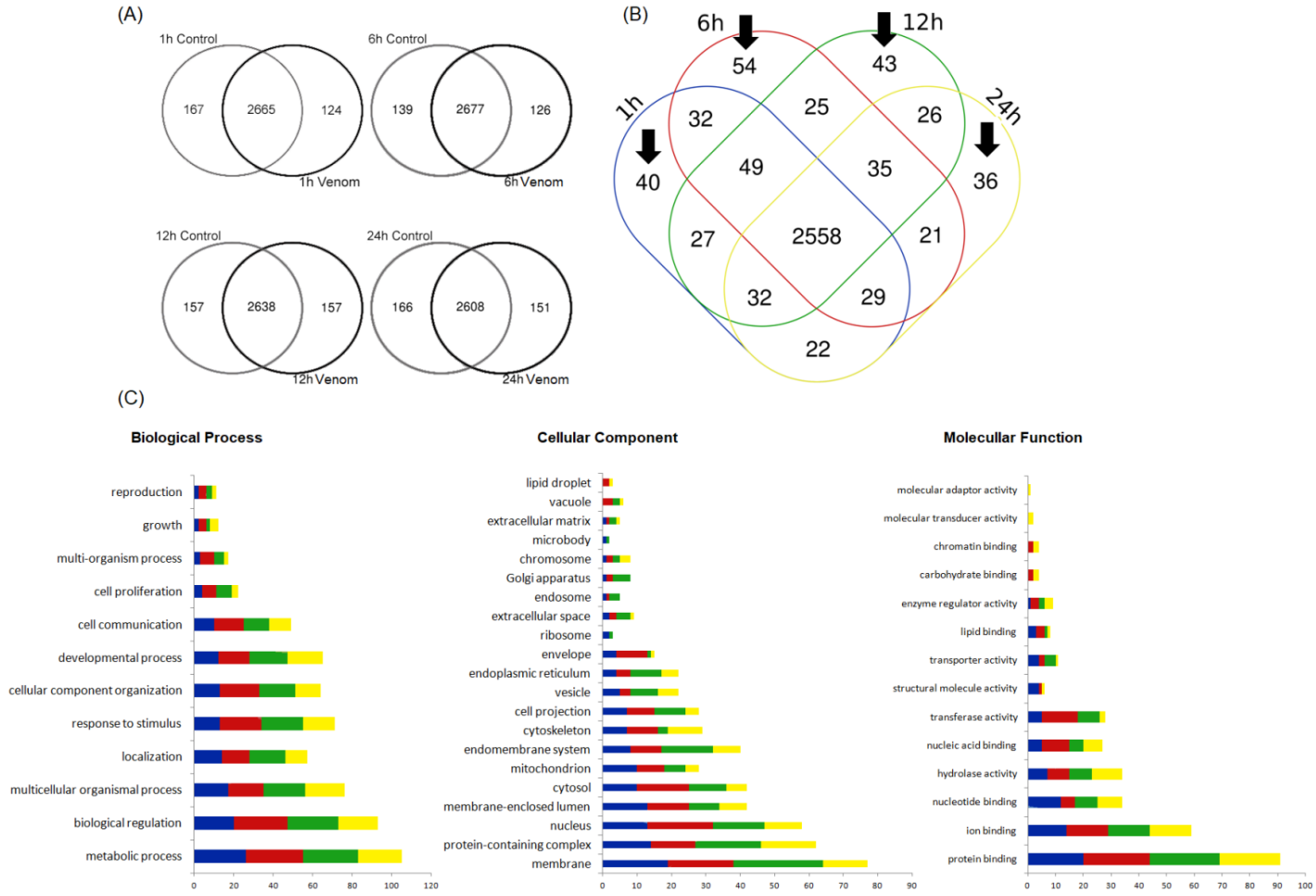


FIGURE 1. Comparison of identified proteins in control and venom treated mice among (A) all proteins identified in the control vs. treated with 0.5 LD *Crotalus durissus terrificus* venom injection at 1 h, 6 h, 12 h and 24 h and (B) all identified proteins at all-time points of mice treated with the rattlesnake venom. Arrows shows proteins exclusively identified in each of the time points. The list of these proteins are presented in supplementary table 2A (C) GO terms for biological processes, cellular component and molecular function of exclusively expressed on mice cerebellum treated with rattlesnake venom (shown in supplementary table 2). N=5 for each condition.

Figure 1A shows proteins that were identified exclusively in the control group or venom treated cerebellum at 1 h, 6 h, 12 h, and 24 h. At 1h post venom treatment, 167 proteins were identified exclusively in the control group and 124 in the venom treated group from a total of 2956 proteins identified. At the 6 h time point 139 proteins were identified exclusively in the control samples and 126 proteins exclusively in the venom treated cerebellums from a total of 2942 proteins identified at this time point. At the 12 h post-venom injection time, corresponding to the most common timeframe for patients to seek for medical treatment after snakebite [19] 157 proteins were identified exclusively in both control group and venom treated group from a total of 2952 proteins identified. At the 24 hours time point, it was identified 166 proteins exclusively in the control group compared to 151 proteins exclusively identified in the venom treated group from a total of 2925 proteins identified at this time point (each of these proteins lists are shown in supplemental table 2a). When proteins from all venom treated groups from each time point were compared (figure 1B) we were able to identify 40 proteins exclusively expressed at 1 h, 54 at 6 h, 43 at 12 h and 36 at 24 h post venom treatment (Table 2).

Proteins from each of these exclusively identified groups in each of the venom treatment time points were analyzed based on Gene Ontology terms for biological processes, cellular component and molecular function in order to identify enrichment of specific terms (Figure 1D). Biological processes showed no particular enrichment for any of the specific terms. Although few cellular components were exclusively identified for ribosome (1 h and 6 h), microbody (1 h and 12 h) and liquid droplet (6 h and 24 h), data didn´t reveal any significant enrichment of any specific category. For the molecular function analysis, a slight exclusivity of molecular adaptor and transducer activity (1 h) and chromatin and carbohydrate binding (6 h and 24 h) was observed.

3.3 Non-Ranked enrichment

Non-ranked enrichment analysis showed that exclusively identified proteins on venom treatment condition are part of different biological process, cellular component or molecular function. In order to distinguish proteins up- and down-regulated from

both control and venom treated conditions, we focused on the over-representation enrichment analysis that highlighted some distinct biological processes, molecular functions and cellular components. At 1 h post-venom injection we observed a down regulation of symmetric synapse (inhibitory synapses), positive regulation and modulation of inhibitory postsynaptic potential. Oxidoreductase activity acting on the CH-CH group of donors, cellular response to increased oxygen levels and membrane hyper polarization were up regulated. A general profile at the 1h time point regarding all down regulated terms plus the hyper polarization of the membrane shows a possible culmination to inhibitory synaptic signaling inhibition.

At the 6 h time point, downregulation of MAPK3 signaling and mitotic G2 DNA damage checkpoint were observed. On the opposite side, neuropilin binding was up regulated. It is known that these terms are all involved in the maintenance of the organism, and therefore they may be related to a possible damage response to the tissue at the short to medium terms.

Moreover, at the 12 h time point, terms involving cellular/ structure development takes place in down regulated terms such as forebrain neuroblast division, intercellular canaliculus, lateral ventricle involvement and neuroblast division. Curiously the regulation of intestinal absorption were also down regulated and interestingly, establishment of the blood-brain barrier were part both of up and down regulated terms, that might indicate a perturbation and at same time an organism response to a damage on the barrier, that may be the action of gyroxin after transposing the blood brain barrier [16].

At the 24 h time point, we observed a different pattern of enrichment of terms compared to other time points. We observed a downregulation of myosin filament, microfilament motor activity, myosin complex and motor activity and an increase of abundance of proteins related to response to endogen compounds such as metal ions and nitrogen compounds. The downregulation of nitrogen compounds response and increase of metal ion response might be related to oxidative stress. One of the clinical manifestations observed upon envenomation is rhabdomyolysis in which the damage in the muscular tissue release myoglobin in the bloodstream that leads to renal failure as a consequence to oxidative stress [35]. Therefore, it is possible that

due to this oxidative stress, the affected muscular tissue releases free iron in the bloodstream that catalyzes ROS increasing the redox imbalance on kidneys [35] sending this signal to the brain through bloodstream.

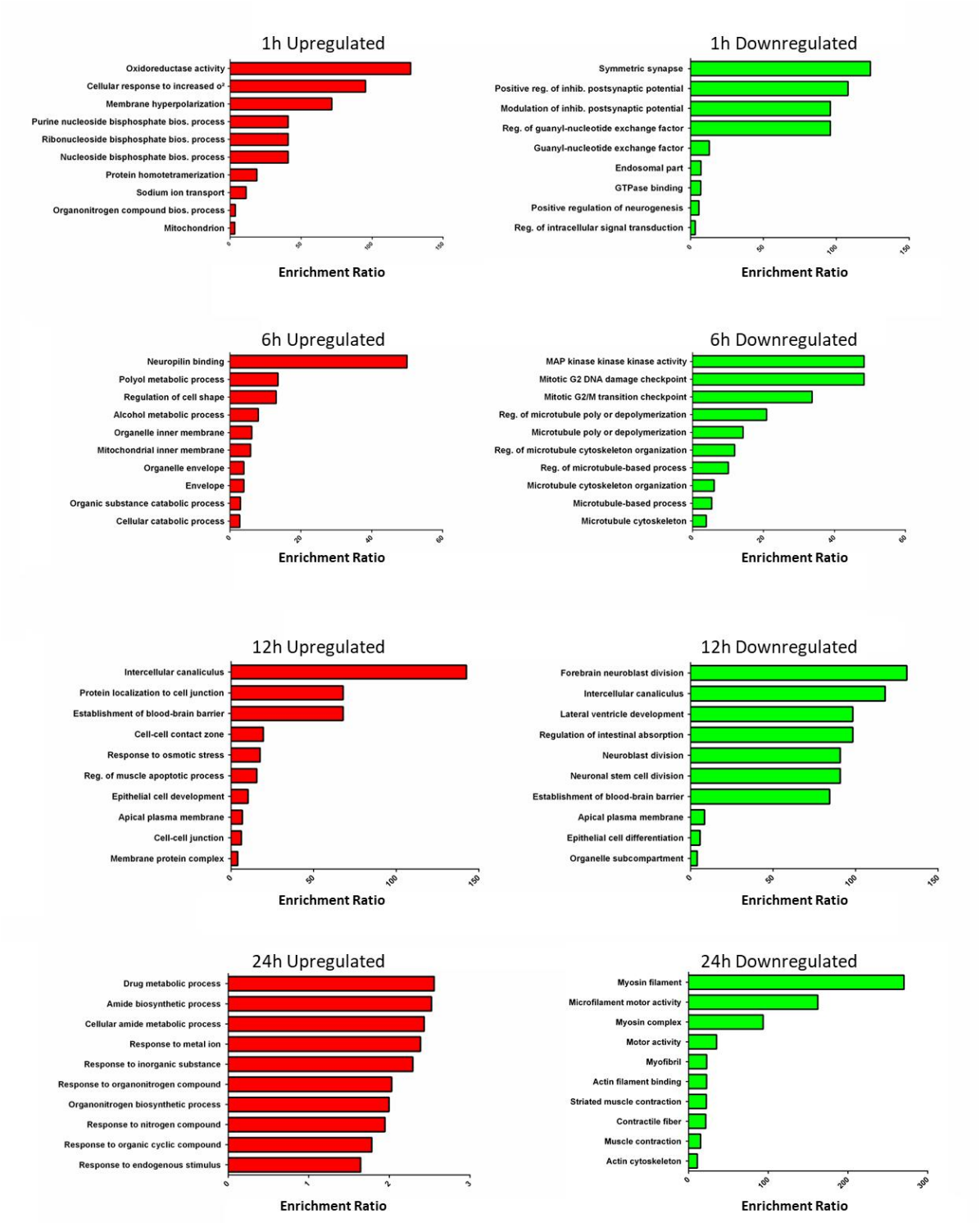


FIGURE 2. Over-representation enrichment analysis using WebGestalt tool. The enrichment was performed using exclusively identified proteins at each time point. The graph summarizes GO biological process, cellular component and molecular function.

3.4 Hierarchical clustering analysis

The log2 fold change data at time points were plotted into a heatmap to compare the changes in clustered proteins during the envenomation process (Figure 3A). The Euclidean clustering analysis grouped the entire data in 6 clusters (Figure 3B) and its GO cellular component was displayed in graphs (Figure 3C). In the first cluster (marked in grey) 49 proteins were up regulated after the *C.d terrificus* venom injection after 1 h, 12 h, and 24 h and decreased at 6h. These proteins are mainly related with mitochondrion and cellular respiration processes. Therefore, at 6 h their decrease suggest that the organism were suffering major disturbances in respiration related processes and at the later time points, the up regulation of such proteins were due to the organism reacting to the stress caused by this perturbation.

In the second cluster (marked in light blue) the highlighted 43 proteins increases in abundance from 1 h to 12 h, until 24 h time point. This particular cluster is in line with the occurrence of oxidative stress in the cerebellar tissue, where proteins such as NADH, represented in this cluster is an important coenzyme for oxidative phosphorylation processes and plays fundamental role in brain function and also in case of dysfunction neuronal diseases [36].

The third cluster (marked in dark red) is represented by 11 proteins, which deceases at 12 h and shows a little growth at 24 h time point. This small cluster interestingly shows proteins related with protein location and apoptosis, which appears to increase over the time, until the 24 h time point.

The fourth cluster (marked in dark yellow), containing 12 proteins, shows an opposite behavior compared to the second cluster, where protein abundances decreases from 1 h to 24 h time point. Proteins in this cluster were enriched for mitochondrion organization, chaperone-mediated protein complex assembly and response to aluminum ion.

At the fifth cluster (marked in blue) containing 131 elements shows proteins that increase in abundance until 12 h and a decrease at 24 h. Enriched proteins in this cluster were related to mitochondrial part and matrix, and myelin sheath that corroborate previous data in figure 2 that shows blood-brain barrier terms

disturbances. Cerebellar tissue might be prone to be directly affected by molecules that increasingly affected the myelin sheath in 24h.

The sixth cluster (marked in purple) containing 95 proteins showed a constant decrease of protein abundance along the time and an abrupt decrease at 24 h. This cluster enriched proteins related to mitochondrion, respiratory chain complex and mitochondrial respiratory chain. Interestingly, it is known that there is a relationship between venom and mitochondrial respiratory chain that are reduced after the envenomation process of diverse animals [37, 38].

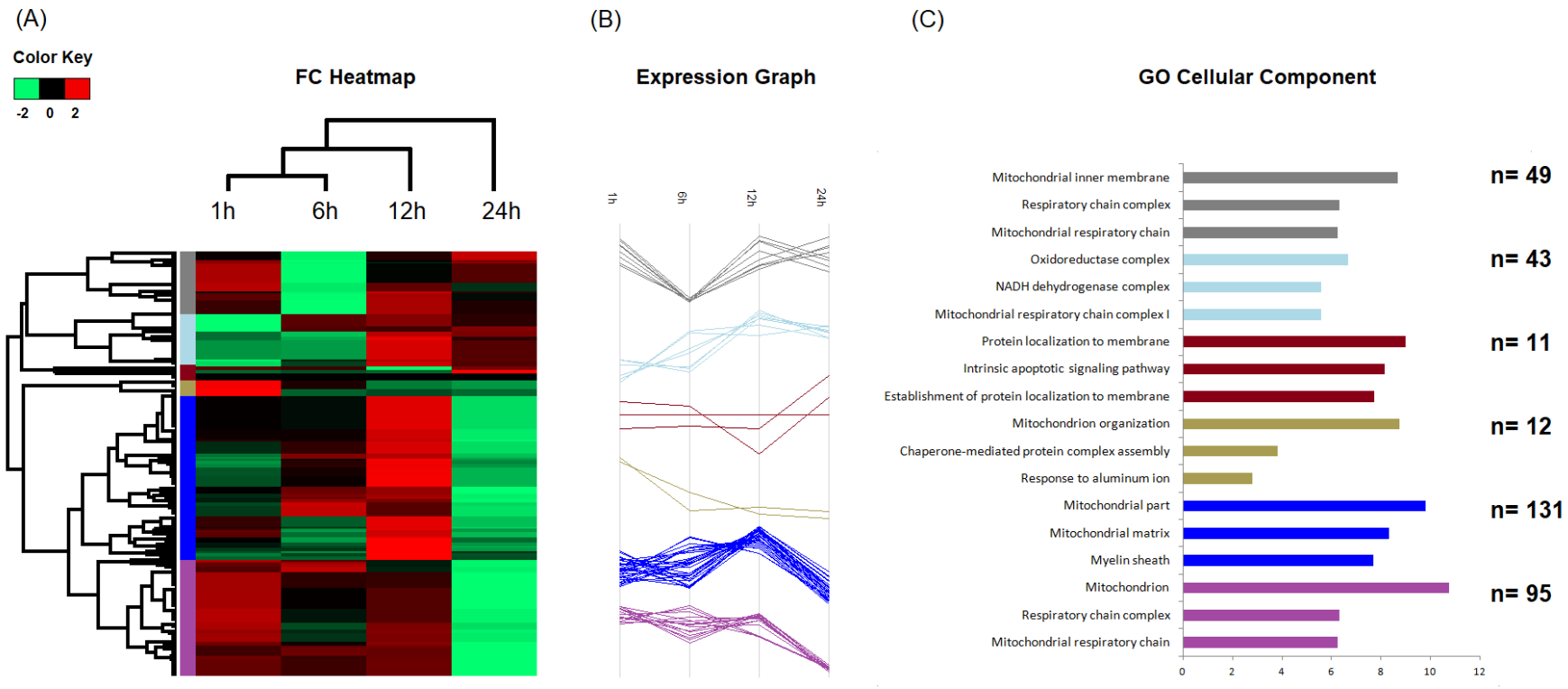
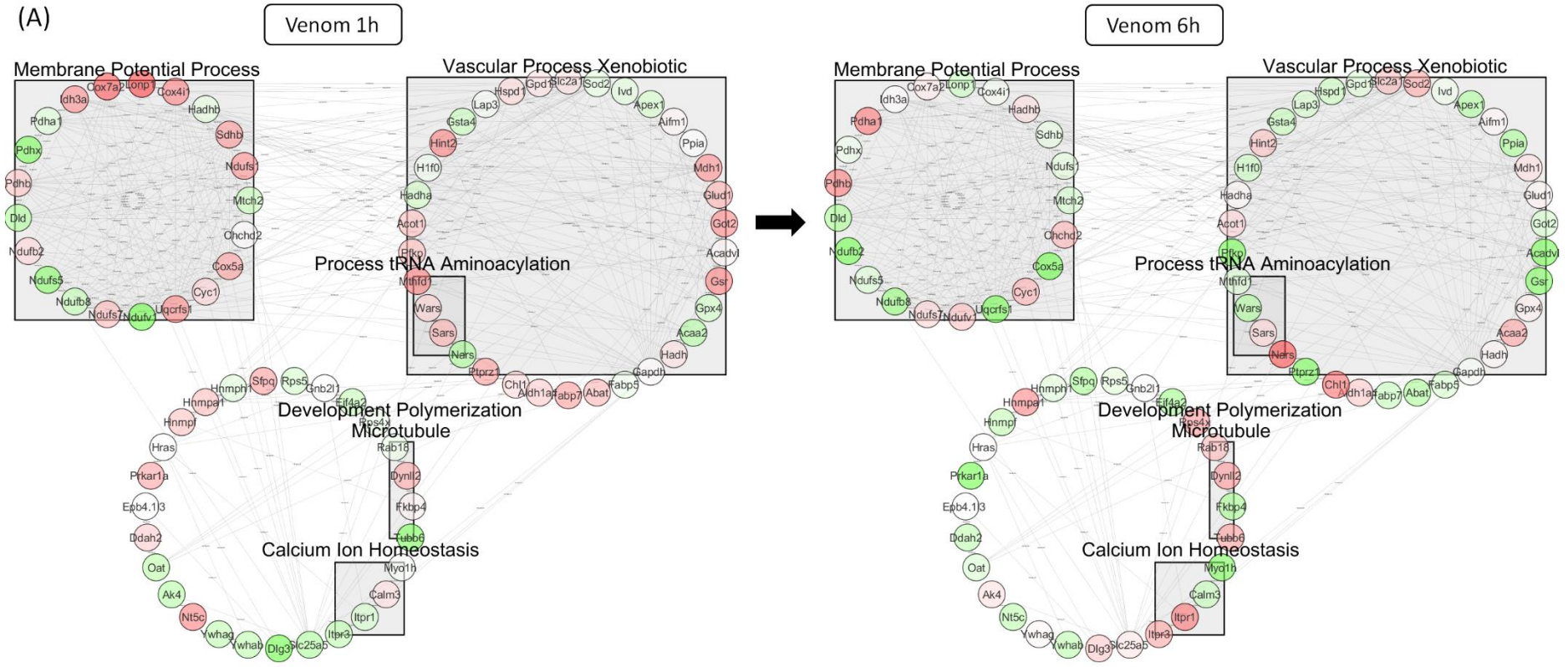


FIGURE 3. Hierarchical clustering of protein quantification levels in the Cerebellum at 1 h, 6 h, 12 h, and 24 h after *C. d. terrificus* venom injection in mice. (A) Heatmap presentation of a hierarchical cluster of most significant proteins detected with quantification in at least two replicates of each treatment time showing the protein behavior changes along with the time. Protein fold change is represented in log2 scale. Each color at the left side of the heatmap represents protein hierarchies represented in the expression graphs and their GO component cellular enrichment. (B) Expression graphs of each cluster. The protein quantification values were normalized by Z-score by rows. (C) GO Cellular component enrichment for all clusters and numbers of proteins composing the cluster.

3.4.1. Network Analysis

The clustering data and the abundance changes over the time were further compiled with network analysis that showed the top five enriched GO terms: the vascular xenobiotic process (24 proteins), membrane potential process (21 proteins), calcium ion homeostasis (4 proteins), development polymerization microtubule (4 proteins) and tRNA aminoacetylation process (4 proteins) (Figure 4). Moreover, we were able to highlight the specific protein abundance variation along with the time from 1h to 24 h in each network clustered group.

In general, it is possible to observe a no-change, increase or decrease in abundance of several proteins from 1 h to 6 h in each of the five highlighted GO terms even of proteins within each of the terms. However, at the 12 h time point, most of the proteins are up regulated, and at the 24 h time point, most of these proteins down regulate.


(A)
Venom 1h
Membrane Potential Process
Vascular Process Xenobiotic
Process tRNA Aminoacylation
Development Polymerization Microtubule
Calcium Ion Homeostasis
Venom 6h
Membrane Potential Process
Vascular Process Xenobiotic
Process tRNA Aminoacylation
Development Polymerization Microtubule
Calcium Ion Homeostasis

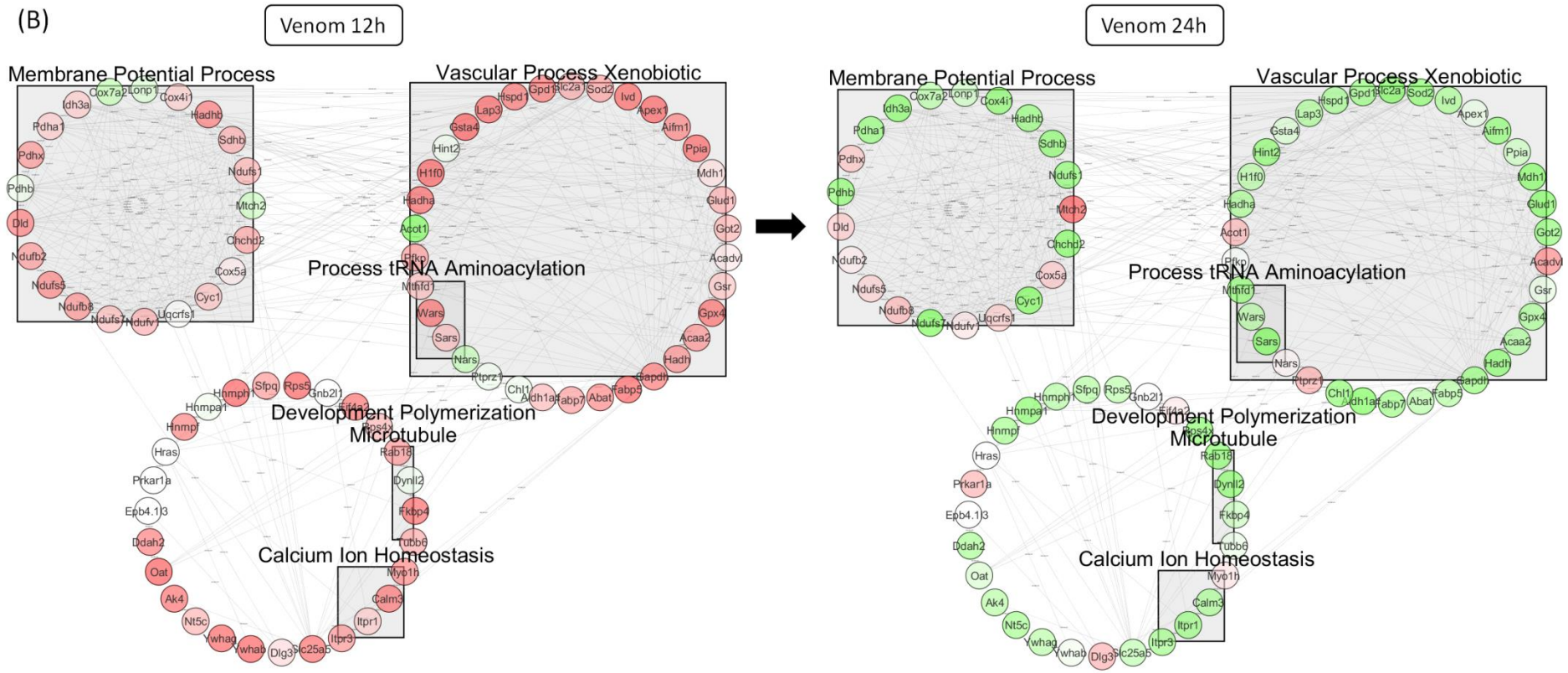


FIGURE 4: Network vision of total profile of protein fold change. The PPI was done using String tools in its own software database in default options on *Mus musculus* database. The PPI information was added on Cytoscape software and network views were created clustering proteins from top 5 GO enriched Biological Processes. The rectangle shows the GO biological process enriched proteins.

4. Discussion

The treatment of mice with 0.5 LD50 of *C.d.terrificus* venom and the observation of proteomic variation from 1 h to 24 h showed molecular changes over the time in the cerebellum after the venom inoculation. Several proteins were exclusively observed in control group injected with saline or in the venom injected mice (Figure 1), such as Dystrophin (106.25 -10logp), that plays a role in the neural maintenance and synaptic signaling [39]. It was also reported to be affected by several snake venoms [40-42], Regulating synaptic membrane exocytosis protein 2 (75.25 -10logp), that have functions on dendrite formation and Neogenin (68.04 -10logp) that have diverse functions on cell, and is also related o neural development and chemorepulsive response [43]. Other than that, we have found proteins that were exclusively found on treated condition, such as Neuropathy target esterase protein (62.22 -10logp) that have been reported to be the target of organophosphorous esters (OP), which causes in man and other vertebrates delayed neuropathy, with several consequences such as, paralysis of limbs, degeneration of long axons and peripheral nerves [44], Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 (65.83 -10logp), that play a role in both heart and brain pacemaker activity [45] and Clusterin (42.73 -10logp), that plays a role of an extracellular chaperone that prevents aggregation of non-native proteins [46] and is it know that its increased expression is involved with brain ischemia [47]. This shows that the abundance changes of some specific proteins showed cases that are in line with the clinical and already described biochemical envenoming process data. All these proteins identified exclusively were analyzed in further non-ranked enrichment in figure 2.

At the 1 h time point (figure 2) we observed an enrichment of terms involved with oxidoreductases that acts on CH-CH2 group of donors that use oxygen as a receptor and a response to increase of oxygen levels that might indicate oxidative stress events that might have initiated in the cerebellar tissue. The events of oxidative stress have been reported mainly due to the nephrotoxicity that is the result of rhabdomyolysis, and acute renal failures induced by the rattlesnake venom [48]. Yamasaki and colleagues [35] have already described the oxidative stress caused by

C. d. terrificus venom in mice, in which concentrations of 80 % of the crude venom were able to cause oxidative stress on cortical and medular tissue. Also in the same context, Melendez-Martinez and colleagues [49] have recently described rattlesnake *Crotalus molossus nigrescens* venom inducing oxidative stress on human erythrocytes upon venom treatment for 24 h.

Biological functional terms enrichment analysis of each protein group showed enhancement and diminishment of terms along with the time at each time point giving us a clue on how *C. d. terrificus* venom injection modulates biological functions from 1 h to 24 h.

Enrichment of terms at 1 h shows that the venom might be, directly or indirectly affecting neuronal transmission of signals in the cerebellum. For example, the enrichment of membrane hyperpolarization might be affecting the synaptic signalization and the downregulation of terms involved with the symmetric synapse, positive regulation of inhibitory potential and modulation of inhibitory post synaptic potential shows that the venom is acting preferentially on symmetric synapses, which regulates the sensory information that comes through the spinal cord, avoiding hyper excitability of the neuronal cells.

At the later 6 h time point we observe an enrichment of neuropillin neural receptor binding. The protein class that binds the most in this particular type of receptor is the sematoforins [50] which are proteins that guide and prevent neurons to move to non-appropriate areas in the neural tissue thus having an important role in the neural development [51]. Since these proteins are strongly related to maintenance of the neural health, we speculate that their up regulation in the neural tissue might be an indicative of a response from the tissue due to the venom-related damage. Several reports have shown the neurotoxicity effects of the *C. d. terrificus* venom or some of its components on synapses and the central nervous system [52]. The down regulation of terms that involves MAPK3, checkpoint on G2/M and mitosis on the G2 phase indicates possible damage to DNA, and the down regulation of these processes on the cell cycle might indicate a reduction of kinases, compromising the entire DNA repair mechanism. In fact, the cytotoxic effect of crotoxin, the major component of *C. d. terrificus* venom on DNA damage has already

been reported by Muller and coleagues when exploring the crotoxin cytotoxic potential in different tumor cell lines [53].

At the 12 h time point we observed the enrichment for blood brain barriers establishment terms, which means that while some molecules are disturbing the barrier´s homeostasis, the organism is trying to repair the damage. It has been previously reported that gyroxin, another important constituent of the *C. d. terrificus* venom is able to transpose the blood-brain barrier [16]. Therefore, gyroxin, or some other venom constituent might be playing a role in this process transposing the brain barrier and causing direct damage in the cerebellum.

Terms altered at the 24 h time point seems to be related to muscle maintenance. In fact, rhabdomyolysis has been reported in patients upon *C. d. terrificus* snake bite [54]. In addition, we speculate that another *C. d. terrificus* venom component known as crotapotin, the secondary subunit of crotoxin, might be playing a role in this process. Crotapotin is known to be myotoxic and cardiotoxic [10, 12] affecting heart and muscle contraction [55]. In addition, some reports suggest that it can also act as a chaperone [56]. Although it doesn´t necessarily means that it allegedly affect muscle and heart by compromising the cerebellar structure, crotapotin might be transposing the brain blood-brain barrier playing a different role in cerebellar structure other than muscle damaging.

Network and clustering analysis of the most significant biological processes alteration based on the quantitative analysis showed disturbances in xenobiotics vascular process response, membrane potential process and tRNA aminoacylation process, with the most important changes at 12 h and 24 h. The overview of the abundance change shows a general increase of protein abundances at 12 h and a decrease of protein abundances after 12 h at all highlighted GO terms.

Focusing on the membrane potential related proteins, we highlight proteins that are members of the cyclooxygenase (Cox) c family, such as, Cox7a2 and Cox4i1 that in normal conditions, contribute for the maintenance of synaptic activity, memory consolidation and hyperemia [57]. In addition, these proteins also play an important role in neuroinflammation [58]. The process of envenomation is normally characterized by a generalized inflammatory state where the normal reaction

prompts to a protective response to the venom. Although in some cases, the response to envenomation may lead to sepsis [59], the organism is able to counterbalance the immune response due to the envenomation. Several works has described the effect of the inflammatory response modulation and hyperalgesia by *Bothrops* and *Crotalus* snake venoms [60, 61]. Interestingly enough, the use of corticosteroid medication has been proposed to increase the efficacy of antivenom therapy in combination with Dexamethasone in experimental *Bothrops atrox* envenomation [62]. Although the rationale for this treatment was focused on eicosanoids, the major mediators of the inflammatory edema induced by *Bothrops* venoms, it was successful on reducing the myotoxic and inflammatory effect induced by the venom of *B. jararaca, B. jararacussu* [63]. Hernandez Cruz and colleagues [64] have described the pro- and anti-inflammatory characteristics of *C. d. terrificus* venom in experimental model envenomation. At the early time points after intraperitoneal venom injection of BALB/c mice, authors observed an increase of serum proinflammatory cytokines such as IL-6 within minutes post venom administration, TNF-α showing a peak at 2 h after venom administration and IFN-g decaying after 4 hours. At later time points after 4 h post venom administration however, they observed a shift in the balance and an increase of anti-inflammatory cytokines such as IL4 and IL10.

Therefore, the increase of Cox family proteins such as Cox7a2 and Cox4i1 in the cerebellum within 6 h after venom injection and decrease until the 24 h time point may indicate a start and control of an inflammation process that is in line with the pro- and anti-inflammatory responses observed by other authors.

In this same cluster, we can find ubiquinone oxidoreductases subunits family of proteins (Ndufs) that upregulates at12 h and abruptly down regulates at 24 h. Mutations affecting the NDUFS gene result on mitochondrial complex I deficiency that is responsible to a wide range of clinical manifestations from lethal neonatal disease to adult-onset neurodegenerative disorders such as Liegh syndrome [65], which causes several neurological disorders such as psychomotor and mental regression [65-67], Leber’s hereditary optic neuropathy [68, 69], and some forms of Parkinson’s disease [69]. Although the observation of this protein family’s modulation

has not yet been described in the process of snake envenomation and more specifically to the *Crotalus* family envenomation, further investigation linking the neurotoxic effect of the *C. d. terrificus* venom to the modulation of these proteins may reveal the exact role of venom action in the brain.

From the calcium ion homeostasis cluster we highlight Itpr1 and Itpr3, which down regulates at 1 h and 24 h, but up regulates between 6 h and 12 h. These proteins are known to play a role in apoptosis mediated by ER stress that releases calcium ions in cytoplasm [70-72]. It is also known that Itpr1 downregulation is related with ataxia, that is a condition were the individual develop severe disturbances on the cerebellum and consequently harms voluntary movements and body balance. Several studies have shown that venoms in general such as from the scorpion *Tityus serrulatus* induces ataxia in individuals alongside other symptoms observed in individuals bitten by *C. d. terrificus*, such as rhabdomyolysis and acute kidney injury [73]. Works related to the same issue has also pointed that ataxia may be associated to crotalic accidents, and the disturbances observed on Itpr proteins may be a clue for the molecular path of this severe damage caused by *C. d. terrificus* envenomation process [74, 75].

Calmodulin 3 (Calm3) has also shown similar modulation to Itpr proteins however, a peak of upregulation at 12 h time point after venom inoculation. Calmodulin is a protein that participates in signaling pathways of a wide variety of biological processes such as proliferation and growth and is a primary Ca2+ sensor in eukaryotic cells [76]. Although Calmodulin is generally considered as an intracellular protein, it has been described that it may play a role in neurotoxicity process by being the receptor for neurotoxins internalization such as PLA2 [77] and may play a role in the inhibition of neurotransmission by associating with snake venom PLA2 in the nerve terminal [78, 79]. Therefore, the observation that after 6 h upon envenomation, the increase of Calm3 in cerebellum may be linked to its binding to *C. d. terrificus* venom PLA2 subunit of crotoxin resulting in synapses blockage in the brain.

From the tRNA Aminoacylation group, we highlight Wars, Sars and Nars, which are the tryptophan, serine, and asparagine t-RNA synthetases respectively.

Wars has a role in cytoskeleton organization and in response to stresses. Wars mutation has been described to lead to severe neuropathy [80-83]. Sars has its major role in binding to VEGFA growth factor that inhibits the MYC proto-oncogene binding and its transcriptional factor activity. Nars has been described to play an important role in the catalytic activity. Mutation in this gene has been linked to severe neuropathies, such as combined oxidative phosphorylation deficiency, which affects the grey matter causing severe disorders, Alpers syndrome and Leigh syndrome [84-86]. The network analysis of these proteins does not show synergy among them along with the time. Although Sars and Wars both decrease in abundance at 24 h, while no change of Sars is observed from 1 h to 12 h, Wars decrease in abundance from 1 h to 6 h and increase from 6 h to 12 h. Nars on the other hand, shows an opposite profile when compared to Wars. Although all these proteins are related to some extent to neurological disorders, their exact role in cerebellum upon *C. d. terrificus* envenomation still needs to be elucidated.

Regarding the development and polymerization of microtubule group, all proteins in this group Rab18, Dynll2, Fkbp4 and Tubb6 shows a peak of abundance between 1 h to 12 h and a decrease of abundance after 12 h. This profile shows a possible result of the response to tissue damage caused by the envenomation in early time points.

Tubb6, which is a microtubule constituent protein [87, 88], were observed at reduced abundance at 6 h, 12 h and 24 h. The down regulation at these time points may characterize a possible tissue damage response, corroborating the observation of microtubule polymerization down regulation observed in figure 2. This data may indicate that the snake envenomation can damage the tissue directly, but it may be possible that toxins is reaching the blood brain barrier and acting in a direct form on certain brain tissues, such as the cerebellum.

In addition, Rab 18 was downregulated at 1 h, 6 h and 24 h. This protein is highly expressed in the brain and plays a role in synaptic vesicles function. Its down regulation may disrupt the neuronal cytoskeleton, and its absence has been related to cause abnormal ER structure. It has also been shown that Rab18 down regulation

is related to several neural deficiencies and accumulation of microtubule filaments and in synaptic terminals [89, 90]

Rucavado and co-workers [91] analyzed wound exudate of mice injected intradermally with *Bothrops asper* snake venom and observed a decrease of Rab18, Tubulin family of proteins (Tubb5, Tubb1, Tuba1c) and FKBP3 protein abundances from 1 h to 24 h upon tissue damage and increase in vascular permeability. Local tissue damage may start very early when compared to the damage that might be affecting the brain tissues. Although the exact timing of damage has not been well characterized yet, our data shows the organism response to tissue damage upon *C. d. terrificus* envenomation within the 12 h of venom administration.

The xenobiotic vascular process cluster shows several proteins with a peak of abundance at 12 h and a decrease in abundance after 12 h after venom injection. Most of these proteins are related to fatty acid and amino acids metabolism pathways which may be related to the organismal response to the early metabolism disorders that envenomation may have caused.

Microscopy histology analysis showed no remarkable difference on mice brain injected with venom or with saline although red neurons were observed in two samples at 1h and 6h venom treatment (Supplemental table 5, Supplemental Figure 5). Red neurons are normally found in central nervous system and is an indicative of acute neuronal injury and subsequent apoptosis or necrosis. Despite this finding is not statistical significant, the observation of red neurons in cerebellum might be an indicative that at some cases, the Crotalus snake venom may be acting directly or indirectly on this organ. Moreover, ELISA test using anti - *C. d. terrificus* venom serum against whole cerebellum lysate showed no antibody recognition (data not shown). Although recognition was not observed, a further analysis using specific antibodies against specific components of the venom might be able to reveal if some of the venom component such as gyroxin is indeed participating in the increase of the permeability of the blood brain barrier. Sangiorgio and coleagues [92] injected *C. d. terrificus* venom at 1mg/ Kg in seven young adult female and male dogs weighting 4 to 7 kg and collected macro and microscopic data at different sites and organs. Despite the alterations observed in spleen, liver, kidney and stomach, no alteration in

brain was observed. Also, in a work of Venkatesan et al [93] who injected *Naja naja naja* snake venom on Swiss albino mice at 2 LD50, brain tissue of envenomated mice, no significant changes in the cells except some marked infiltration of leukocytes were seen after 48 h of venom treatment. Although different authors have used different concentration ratio of venom to animal weight, the sub-lethal dose we have used might have not been enough to induce microscopic tissue damage that could be observed in the histology analysis. However, the 0.5 LD50 were enough to induce molecular changes and identify and quantify several proteins modulation along with the time.

Taken together, we were able to identify proteins related to both tissue damage and inhibition of signaling due to *C. d. terrificus* venom action. Furthermore, we were able to observe the up regulation of terms with time that is related to neural cells development and normal cell´s maintenance, showing that the venom might be inducing cerebellar tissue disturbances that might be related to some of the *C. d. terrificus* venom toxins, such as crotoxin, gyroxin and crotamine.

5. Conclusion

The proteomic modulation events in the mice cerebellum after *C. d. terrificus* venom injection in mice gastrocnemius muscle highlight the complex temporal dynamic of the envenomation process in one of the brain regions. The neurotoxicity of this snake venom and its effects at all different brain tissues still needs to be elucidated; however, proteomic data presented in this work have provided insights into the mechanisms of venom inducing tissue damage unraveling novel events that can change perspectives and the way snake envenomation is molecularly described. The molecular modulation observed in the cerebellar tissue caused by *Crotalus durissus terrificus* shows synapses inhibition and perturbation on blood brain barrier maintenance that points to a possible participation of venom toxins as responsible for the actions described in this study. These findings shed new light in the complex mechanisms involved in the inflammatory responses of tissue in snakebite envenomation and points to potential novel routes for therapeutic intervention to attenuate envenomation mortality and morbidity.

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7. No conflict of interest statement

Authors declare that there is no conflict of interest.

Note: The supplemental figures 1-4 and tables 1-4 and all raw data are provided at the Center for Computational Mass Spectrometry of the University of California, San Diego, MassIVE website and can be downloaded from ftp://MSV000084135@massive.ucsd.edu/Montoni_F_et.al/, login: leoiwai, password: Proteoma13

Table 1 – Quantity of total protein extract obtained from each mouse / mg.

	Replicate #					
Sample	**1**	**2**	**3**	**4**	**5**	**Mean**
1h CTRL	0.45	0.52	0.52	0.45	0.51	0.49
1h CDTv	0.44	0.39	0.45	0.49	0.61	0.48
6h CTRL	0.47	0.51	0.48	0.57	0.45	0.5
6h CDTv	0.47	0.48	0.45	0.47	0.23	0.42
12h CTRL	0.45	0.38	0.34	0.38	0.36	0.38
12h CDTv	0.42	0.33	0.35	0.46	0.38	0.39
24h CTRL	0.39	0.42	0.34	0.38	0.42	0.39
24h CDTv	0.39	0.42	0.41	0.41	0.4	0.41

CTRL = control CDTv = *Crotalus durissus terrificus* venom

Figure Legends

FIGURE 1. Comparison of identified proteins in control and venom treated mice among (A) all proteins identified in the control vs. treated with 0.5 LD *Crotalus durissus terrificus* venom injection at 1 h, 6 h, 12 h and 24 h and (B) all identified proteins at all-time points of mice treated with the rattlesnake venom. Arrows shows proteins exclusively identified in each of the time points. The list of these proteins are presented in supplementary table 2A (C) GO terms for biological processes, cellular component and molecular function of exclusively expressed on mice cerebellum treated with rattlesnake venom (shown in supplementary table 2). N=5 for each condition.

FIGURE 2. Over-representation enrichment analysis using WebGestalt tool. The enrichment was performed using exclusively identified proteins at each time point. The graph summarizes GO biological process, cellular component and molecular function.

FIGURE 3. Hierarchical clustering of protein quantification levels in the Cerebellum at 1 h, 6 h, 12 h, and 24 h after C. d. terrificus venom injection in mice. (A) Heatmap presentation of a hierarchical cluster of most significant proteins detected with quantification in at least two replicates of each treatment time showing the protein behavior changes along with the time. Protein fold change is represented in log2 scale. Each color at the left side of the heatmap represents protein hierarchies represented in the expression graphs and their GO component cellular enrichment. (B) Expression graphs of each cluster. The protein quantification values were normalized by Z-score by rows. (C) GO Cellular component enrichment for all clusters and numbers of proteins composing each cluster.

FIGURE 4. Network vision of total profile of protein fold change from 1h to 24h time points. The PPI analysis was performed using String-db tool using in its own *Mus musculus* database. The PPI information was added on Cytoscape software and network views were created clustering proteins from the top 5 GO enriched Biological Processes. The rectangle shows the GO biological process enriched proteins.

Supplemental Tables 1 – 4:

Available at the Center for Computational Mass Spectrometry of the University of California, San Diego, MassIVE website. Tables can be downloaded from:

ftp://MSV000084135@massive.ucsd.edu/Montoni_F_et.al

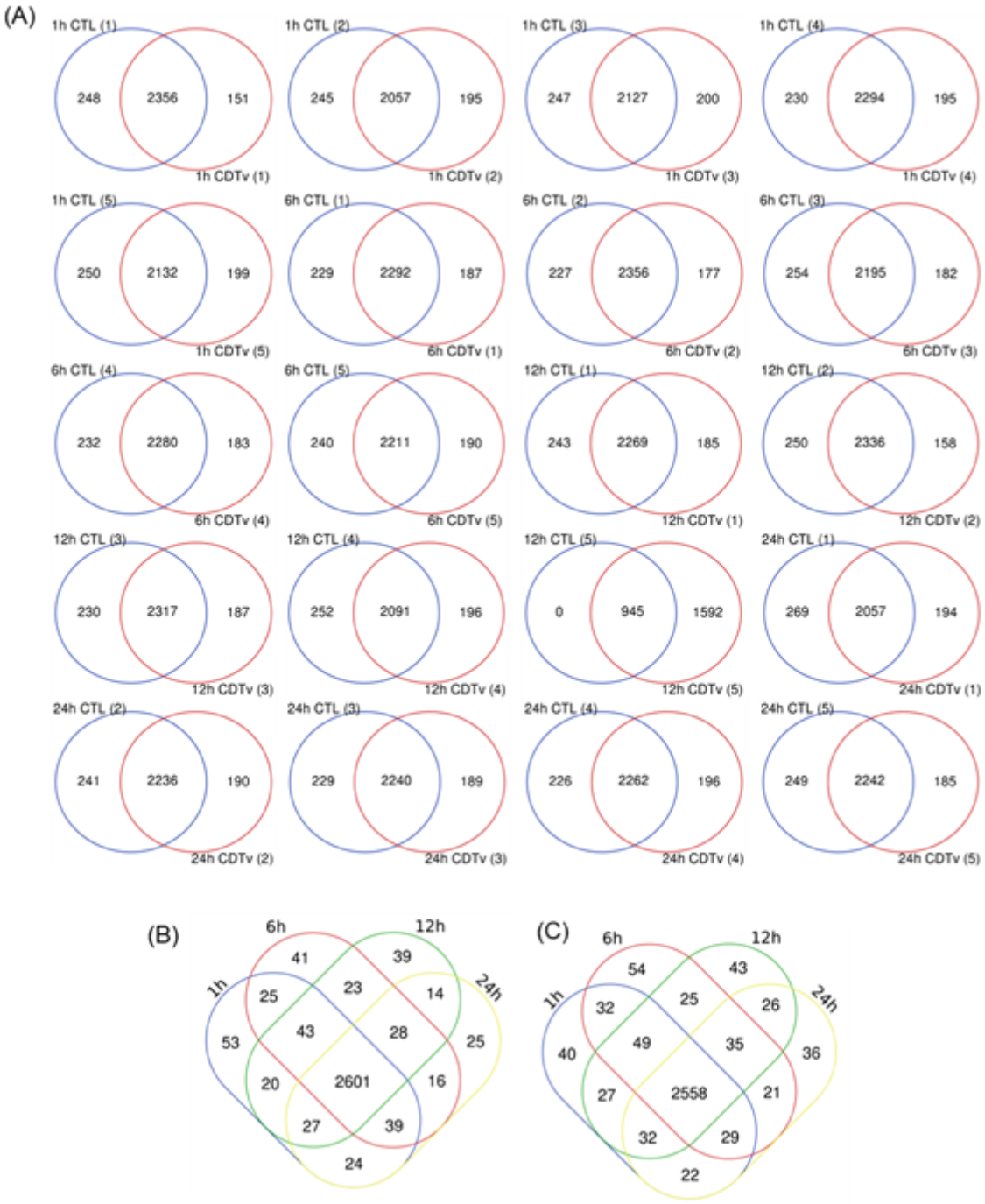
(user login: leoiwai, password: Proteoma13)

Supplemental Table 5 - Hematoxylin Eosin assay: Gliosis

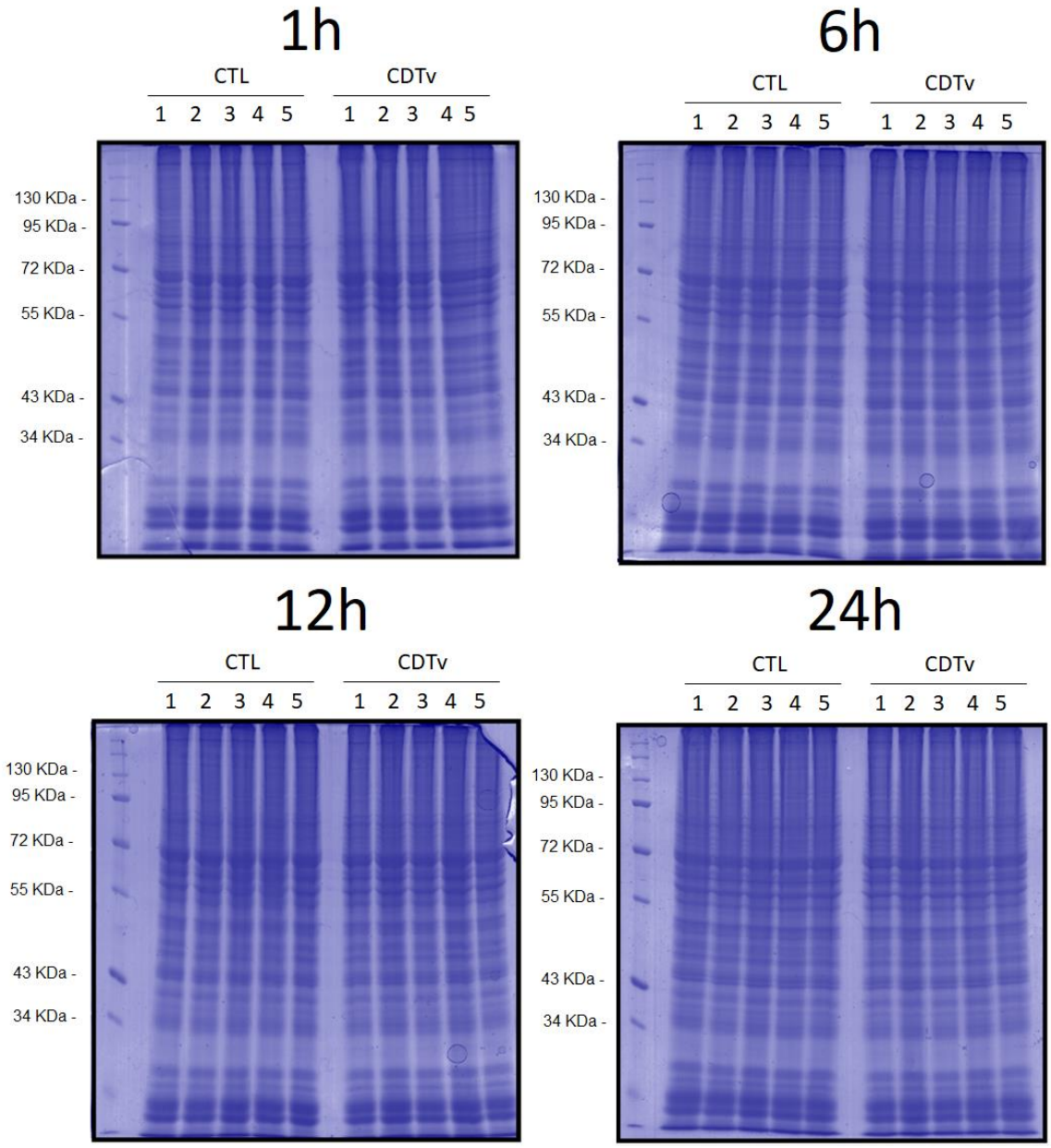
	Control	Venom
1h		XX
6h	X	XX
12h	X	XX
24h	X	X

All observed gliosis points were considered mild indicating that there was no significant change between mice injected with the venom and the control group for gliosis. The 1 h post-injection time was the only time that showed a difference between venom-treated mice and control. Caption: X - Observed in one replicate, XX - Observed in two replicates. N = 3 for each condition

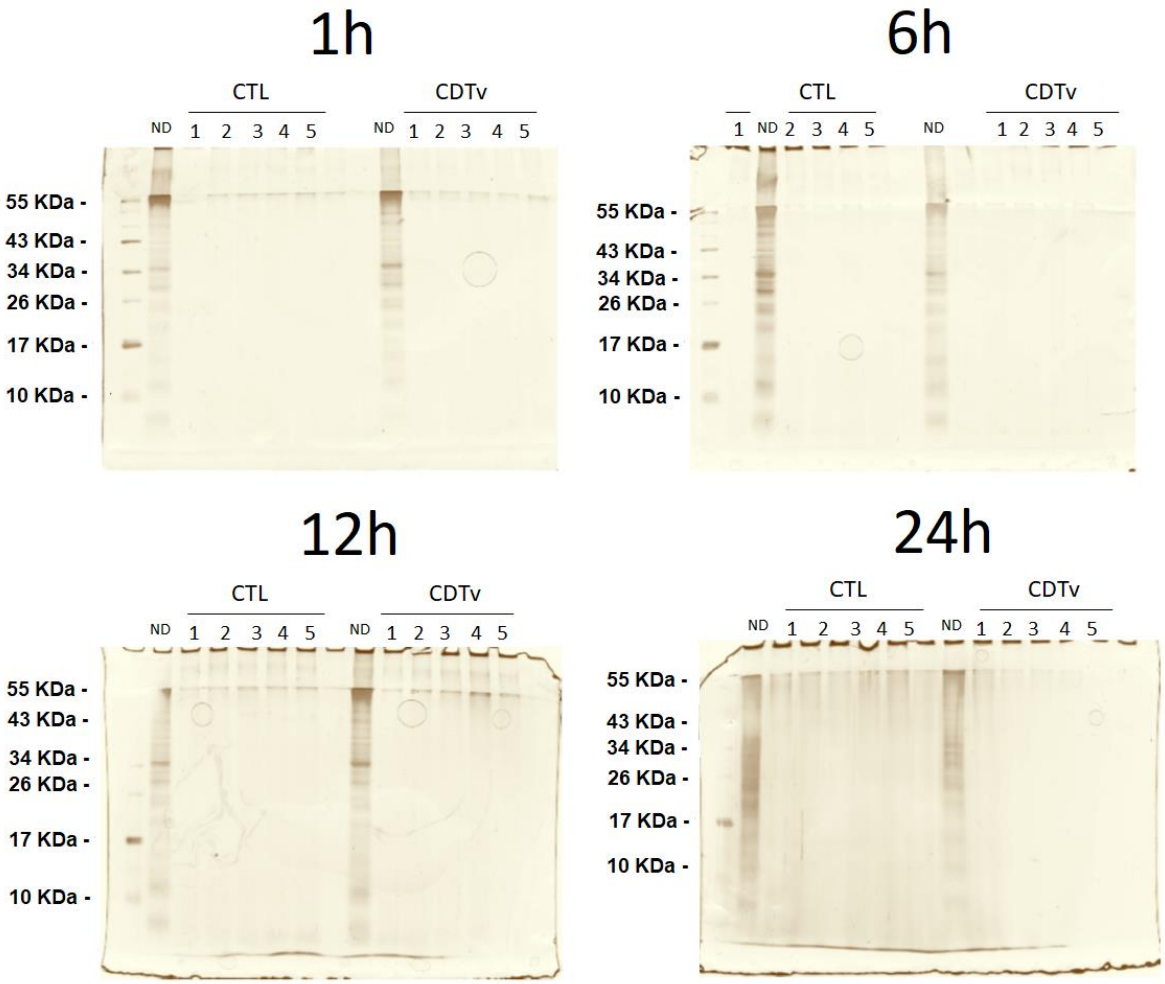
Supplemental Figure 1. Replicate analysis of the identified proteins in each sample group (Control vs venom treated) at each time point (A); Comparison of proteins identified in each time point in the control group (B) and in the venom treated group (C) at each time point indicating number of proteins identified exclusively at each time point and condition. CTL = control. CDTv = *Crotalus durissus terrificus* venom treated.



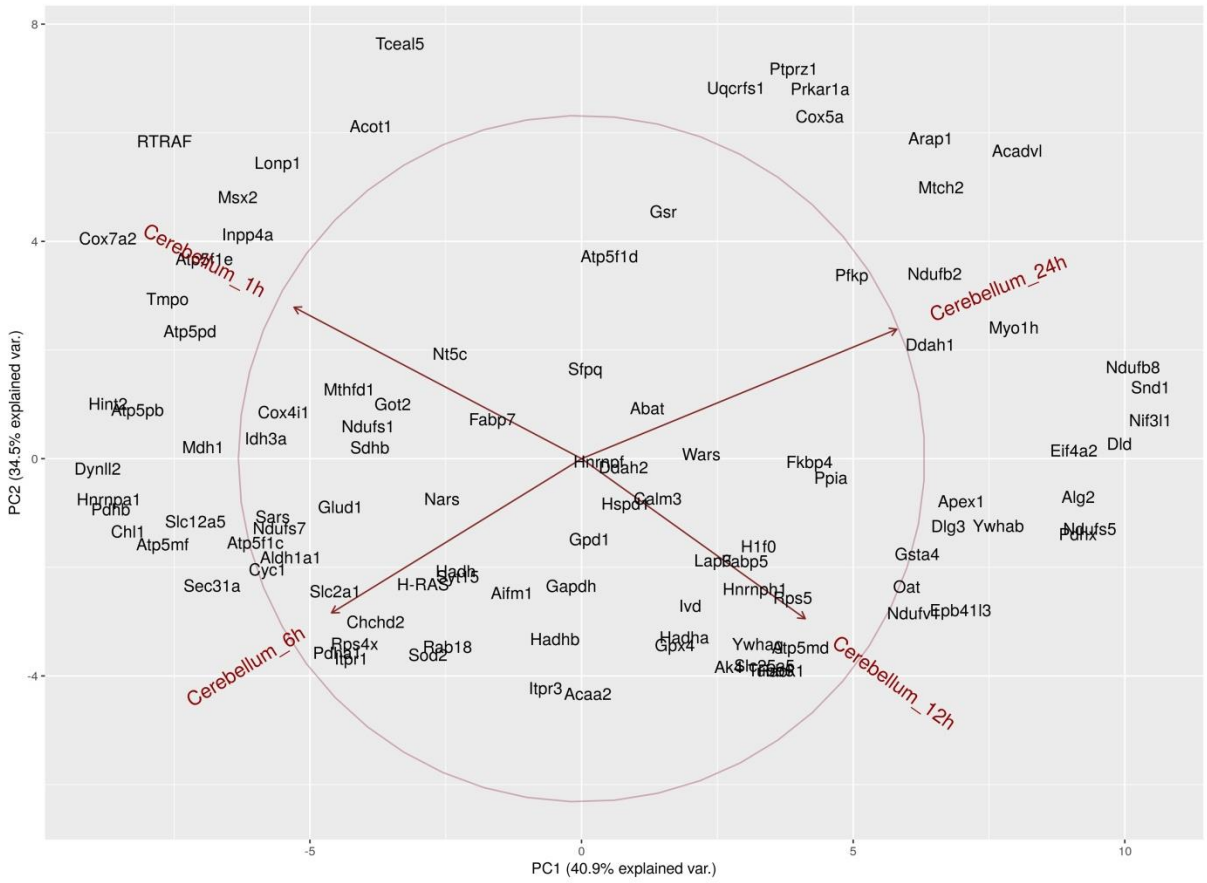
Supplemental Figure 2. Protein lysis quality control analysis by SDS-PAGE stained with colloidal coomassie brilliant blue. An equivalent of 15 □g of samples was applied in each lane in order to observe protein lysis efficiency and check for possible visual difference among different time points. CTL = control. CDTv = *Crotalus durissus terrificus* venom treated. 1-5 = replicate number 1 to 5.



Supplemental Figure 3. Protein digestion quality control analysis by SDS-PAGE stained with silver nitrate. An equivalent of 3 □g of samples was applied in each lane. CTL = control. CDTv = *Crotalus durissus terrificus* venom treated, ND = non digested control. 1-5: replicate number 1 to 5

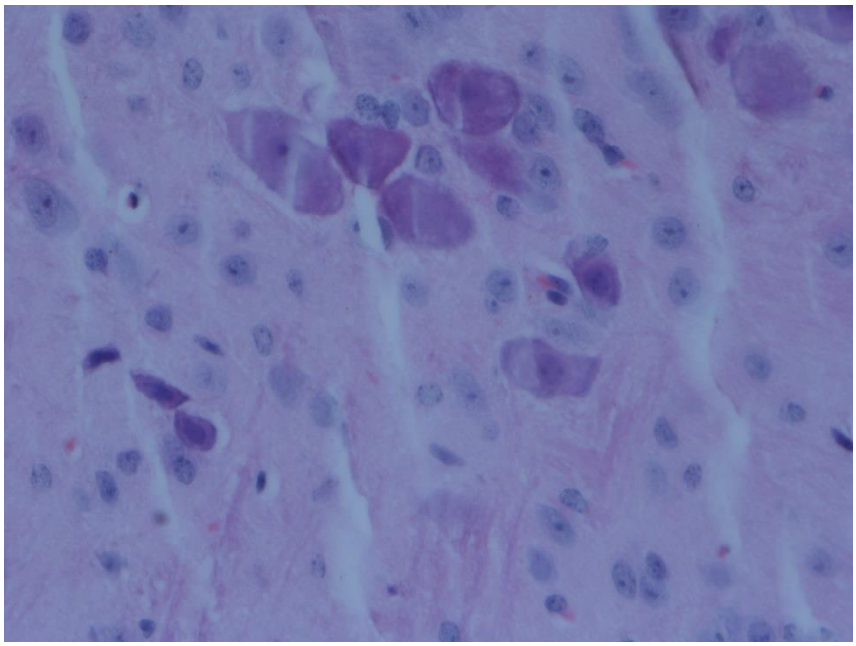


Supplemental Figure 4. Principal component analysis in a 2D graph of PC1 and PC2, which explains 75.2% of the variance. The vectors representing the treatment times are well spaced in terms of the observed proteome indicating that those proteins can be more related for each specific time point and that the vectors that are orthogonal are poorly correlated.

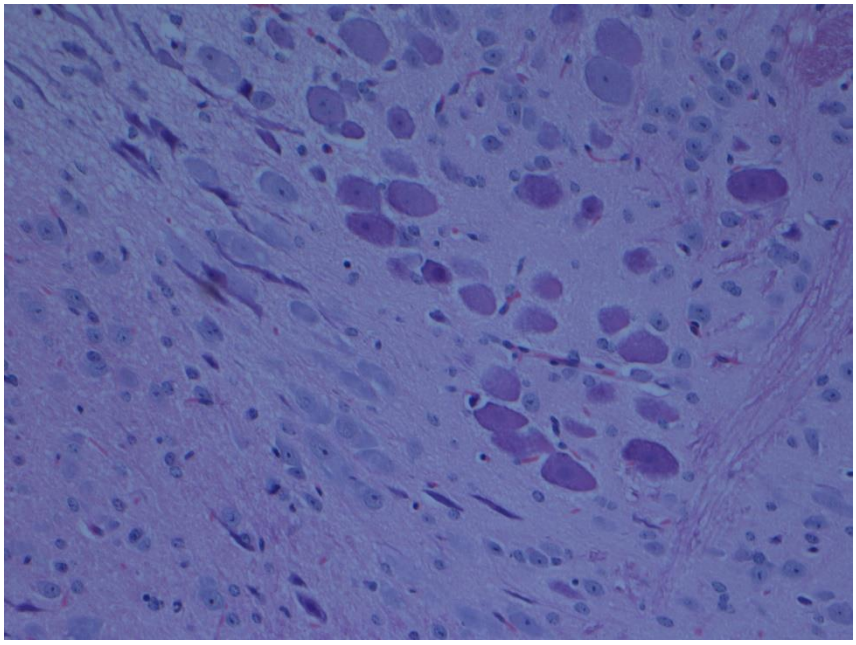


Supplemental Figure 5. Hematoxylin Eosin staining histology analysis. Mice were injected with 0.5 LD50 of the *C.d.terrificus* venom. Red neurons were observed only in one replicate at 1 h (A) and 6 h (B) after venom injection. Although not significant, these data contribute to corroborate the molecular changes observed in proteomic analysis by mass spectrometry.

A.



B.



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4 CONCLUSÕES GERAIS

Os eventos observados nas análises proteômicas do cerebelo do camundongo após a inoculação da peçonha da serpente *C. d. terrificus* no músculo gastrocnêmio permitiu-nos destacar a complexa mudança de níveis de diferentes proteínas ao longo do tempo no cerebelo. Neste estudo pudemos apontar alguns pontos chave que podem ser utilizados para uma maior compreensão do envenenamento á nível molecular. As mudanças proteômicas observadas apontam para dano em sinapses inibitórias, perturbação da barreira hematoencefálica que indicam uma possível participação de não apenas uma, mas diversas moléculas presentes na peçonha da serpente *Crotalus durissus terrificus* seguido de diversos efeitos sistêmicos. As informações obtidas neste estudo podem ser utilizadas como base para a ampliação do conhecimento e também a criação de possíveis aditivos ou novas perspectivas para tratamentos em casos de envenenamento pela serpente *Crotalus durissus terrificus.*

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APÊNDICE

Figura A 1. Comparação da identificação de proteínas entre o grupo controle e tratado com peçonha de *C. d. terrificus* nas diferentes réplicas e diferentes tempos. (A) Comparação de cada uma das condições, (B) Comparação da identificação entre as proteínas identificadas no grupo controle e (C) comparação da identificação entre as proteínas identificadas no grupo tratado com a peçonha.

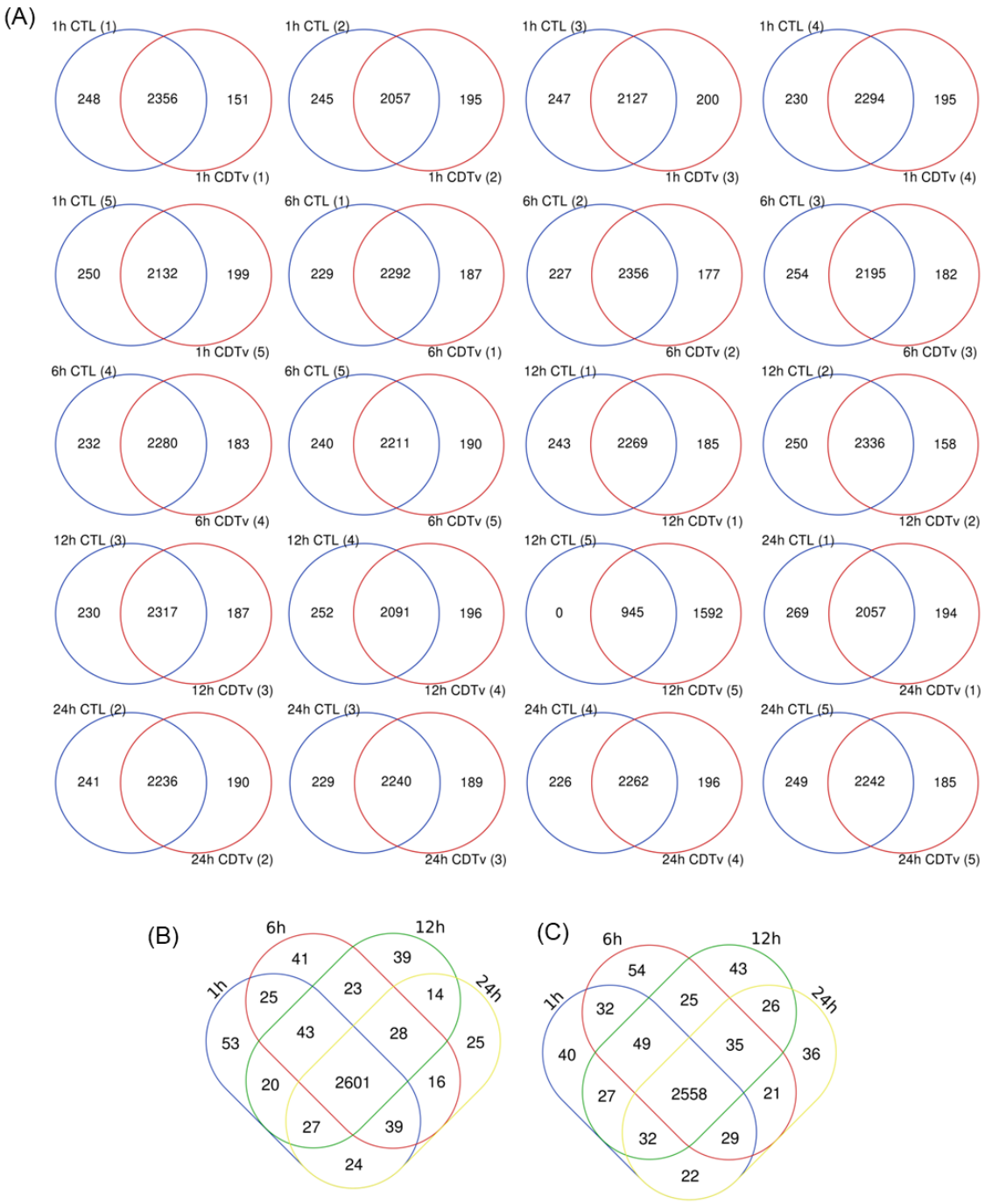
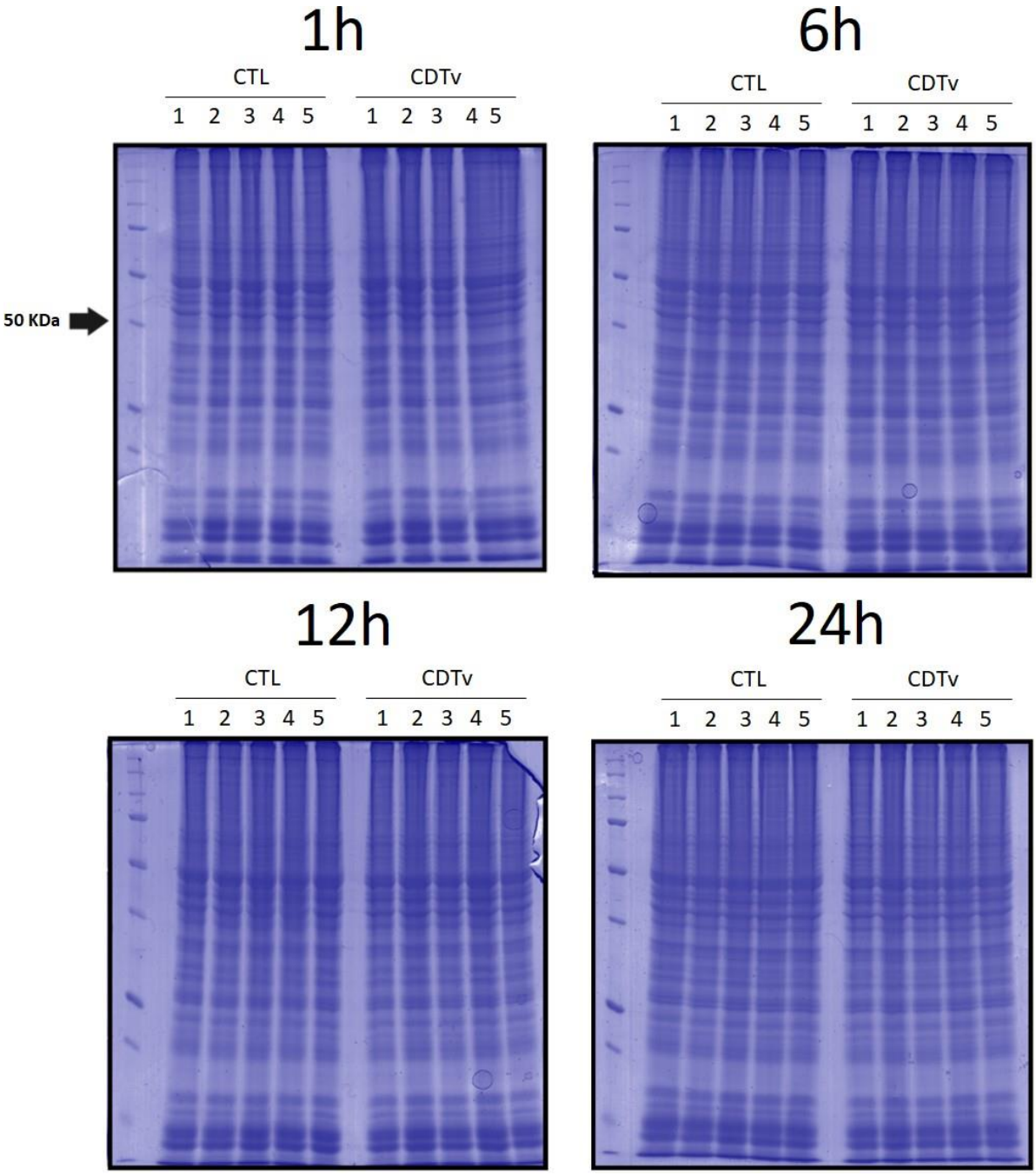
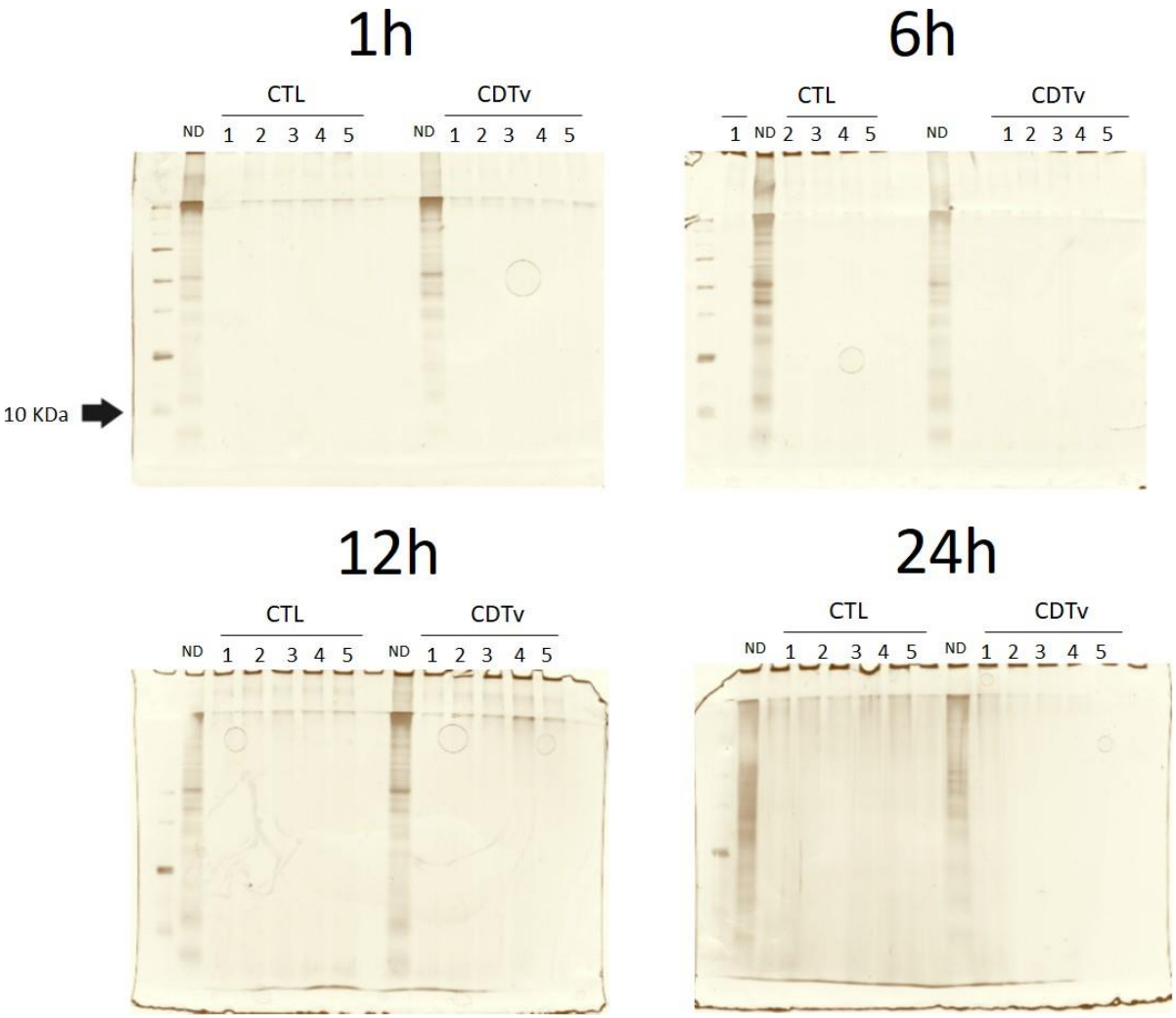


Figura A 2. SDS-PAGE dos lisados de cerebelo de cada uma das réplicas analisadas por espectrometria de massas.



SDS-PAGE corado com *Coomassie* brilliant blue. 15 µg de extrato proteico foram aplicadas em cada um dos *lanes*. Em um primeiro momento, procuramos diferenças entre as réplicas do controle versus tratado com a peçonha, porém nenhuma diferença significante entre os grupos foi observada. Esta análise permitiu observamos a consistência das réplicas após a precipitação de proteínas. CTL = Controle, CDTv = Camundongo tratado com a peçonha da serpente *Crotalus durissus terrificus*, ND = Controle não digerido.

Figura A 3. Controle da digestão tripsínica das amostras a serem analisadas por espectrometria de massas.



SDS-PAGE corada por nitrato de prata. 2 µg de cada amostra foi aplicada em cada *lane*. O objetivo deste ensaio foi verificar a qualidade e homogeneidade de cada amostra a ser analisada por espectrometria de massas comparando os extratos digeridos com não digeridos. Nenhuma das amostras apresentou bandas o que indica a eficiência da digestão tripsínica. CTL = Controle, CDTv = Camundongo tratado com a peçonha da serpente *Crotalus durissus terrificus*, ND = Controle não digerido.