UNIVERSIDADE FEDERAL DO PAMPA
***CAMPUS* SÃO GABRIEL**
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

RAQUEL SOARES OLIVEIRA

ATIVIDADE BIOLÓGICA DO VENENO DE *RHINELLA ICTERICA* (ANURA: BUFONIDAE) SOBRE O SISTEMA NERVOSO DE VERTEBRADOS

DISSERTAÇÃO DE MESTRADO

SÃO GABRIEL, RIO GRANDE DO SUL, BRASIL
2016

RAQUEL SOARES OLIVEIRA

ATIVIDADE BIOLÓGICA DO VENENO DE *RHINELLA ICTERICA* (ANURA: BUFONIDAE) SOBRE O SISTEMA NERVOSO DE VERTEBRADOS

Dissertação apresentada ao Programa de Pós-Graduação *Stricto Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Titulo de Mestre em Ciências Biológicas.

Orientadora: Profª. Drª. Lúcia Vinadé

Coorientador: Prof. Dr. Chárist on André Dal Belo

São Gabriel

2016

Ficha catalográfica elaborada automaticamente com os dados fornecidos pelo(a) autor(a) através do Módulo de Biblioteca dos Sistema GURI (Gestão Unificada de Recursos Institucionais).

O1206a

Oliveira, Raquel Soares
ATIVIDADE BIOLÓGICA DO VENENO DE *RHINELLA ICTERICA* (ANURA: BUFONIDAE) SOBRE O SISTEMA NERVOSO DE VERTEBRADOS / Raquel Soares Oliveira.
83 p.

Dissertação(Mestrado)-- Universidade Federal do Pampa, MESTRADO EM CIÊNCIAS BIOLÓGICAS, 2016.
"Orientação:Drª. Lúcia Vinadé".

1. Qualidade Ambiental. 2. Neurobiologia. 3. Farmacologia. I. Título.

RAQUEL SOARES OLIVEIRA

ATIVIDADE BIOLÓGICA DO VENENO DE *RHINELLA ICTERICA* (ANURA: BUFONIDAE) SOBRE O SISTEMA NERVOSO DE VERTEBRADOS

Dissertação apresentada ao Programa de Pós-Graduação *Stricto Sensu* em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Titulo de Mestre em Ciências Biológicas.

Aos três dias do mês de março do ano de dois mil e dezesseis, às 10:30 horas no campus São Gabriel, realizou-se a prova de Defesa Pública de Dissertação, intitulada: "ATIVIDADE BIOLÓGICA DO VENENO DE RHINELLA ICTERICA (ANURA: BUFONIDAE) SOBRE O SISTEMA NERVOSO DE VERTEBRADOS ", na área de concentração Ciências Biológicas, de autoria da Candidata Raquel Soares Oliveira, aluna do Programa de Pós – Graduação em Ciências Biológicas, nível de Mestrado. A Banca Examinadora esteve constituída pelos professores:

Presidente: LUCIA HELENA DO CANTO VINADÉ

Membros: CARINA BOECK, THAIS POSSER E CHARISTON A. BELO

Concluídos os trabalhos de apresentação e argüição, a candidata foi APROVADA pela Banca Examinadora. Foi concedido um prazo de ( ) dias, para que a mesma efetue as correções sugeridas pela Banca Examinadora no parecer, e apresente o trabalho em sua redação definitiva, sob pena de não expedição do Diploma. E, para constar, foi lavrada a presente ata, que vai assinada pelos membros da Banca examinadora.

Orientador-Presidente: LUCIA HELENA CANTO VINADÉ

1º Examinador: CARINA BOECK

2º Examinador: THAIS POSSER

3º Examinador: _____

Dedico essa dissertação à minha amada mãe Raimunda e meu amado Fabricio, por estarem ao meu lado de modo incondicional e pela paciência que tiveram comigo, ao me ouvir falar tantas horas sobre esse tal de Rhinella.

AGRADECIMENTOS

Aos professores Lúcia Vinadé e Cháriston dal Belo agradeço imensamente os ensinamentos, a paciência, a oportunidade e a confiança depositada em mim.

Aos professores Tiago Gomes, Leandro Lorentz e Cleci Menezes por toda ajuda para a concretização deste trabalho.

Aos professores Carina Rodrigues Boeck, Thaís Posser e Dennis Reis de Assis que cordialmente aceitaram participar da banca examinadora e ofereceram valiosas sugestões.

À UNIPAMPA e ao programa de pós graduação por esta oportunidade, e a CAPES pelo suporte financeiro.

Aos colegas do LANETOX, por toda a ajuda nos dias longos de experimentos, pelos momentos de descontração nas horas vagas e pelos quilos adquiridos com as idas ao "Monstro".

Aos colegas do PPGCB especialmente à Giulianna, Nathane e Matielo por toda a ajuda e disponibilidade.

Ao Tio Carlos e Tio Douglas agradeço a toda ajuda profissional e pessoal, a paciência que tiveram comigo nos dias de drama existencial e por todas as palavras de motivação.

À minha família pela confiança depositada e por todas as formas de auxílio. E a minha família gaúcha, principalmente minha sogra Teresinha por me acolherem com tanto carinho.

Ao meu amado Fabricio por toda a paciência, carinho e apoio incondicional! Essa conquista também é sua!

Às minhas amadas mãe Raimunda e minha tia Sandra por toda a paciência, pela confiança, por sempre me apoiarem, e é claro, pelo suporte financeiro. Sem vocês nada disso seria possível!

À todos vocês meu muito obrigada!

E assim, depois de muito esperar, num dia como outro qualquer, decidi triunfar.
Decidi não esperar as oportunidades e sim, eu mesmo buscá-las.
Decidi ver cada problema como uma oportunidade de encontrar uma solução.
Decidi ver cada deserto como uma possibilidade de encontrar um oásis.
Decidi ver cada noite como um mistério a resolver.
Decidi ver cada dia como uma nova oportunidade de ser feliz.
Naquele dia descobri que meu único rival não era mais que minhas próprias limitações e que enfrentá-las era a única e melhor forma de as superar.
Naquele dia, descobri que eu não era o melhor e que talvez eu nunca tivesse sido.
Deixei de me importar com quem ganha ou perde.
Agora me importa simplesmente saber melhor o que fazer.
Aprendi que o difícil não é chegar lá em cima, e sim deixar de subir.
Aprendi que o melhor triunfo é poder chamar alguém de "amigo".
Descobri que o amor é mais que um simples estado de enamoramento,
"o amor é uma filosofia de vida".
Naquele dia, deixei de ser um reflexo dos meus escassos triunfos passados e passei a ser uma tênue luz no presente.
Aprendi que de nada serve ser luz se não iluminar o caminho dos demais.
Naquele dia, decidi trocar tantas coisas...
Naquele dia, aprendi que os sonhos existem para tornarem-se realidade.
E desde aquele dia já não durmo para descansar...
simplesmente durmo para sonhar.

Walt Disney

RESUMO

Os venenos animais são fontes de compostos bioativos com aplicabilidade terapêutica. Os anuros produzem através de glândulas paratóides, uma secreção venenosa rica em compostos de diversas classes químicas, as quais apresentam uma série de atividades farmacológicas de interesse biotecnológico. Os sapos da espécie *Rhinella icterica* (Spix, 1824), pertencem a um grupo de animais venenosos presentes no bioma Pampa com carência de estudos famacológicos e toxicológicos. Para os ensaios biológicos, os sapos foram coletados na região de Derrubadas, no estado do Rio Grande do Sul. O veneno foi extraído manualmente por compressão das glândulas paratóides, tratado por extração metanólica seguida de liofilização e então foi chamado de MERIV. A neurobiologia do veneno foi avaliada sobre a junção neuromuscular de aves, através da preparação *biventer cervicis* de pintainhos (BCP) e, através da análise das desidrogenases em fatias hipocampais de camundongos. A incubação de MERIV (5, 10, 20, 40 µg/mL) e Digoxina (6,5; 13; 26 e 52 nM) em fatias hipocampais de camundongos, induziram um efeito dose dependente na viabilidade celular. Apenas MERIV (5 µg/mL) e Digoxina (6,5 e 13 nM) provocaram aumento significativo da viabilidade celular de 36 ± 10%, 52 ± 7% e 57 ± 13%, $p<0.05$, respectivamente, enquanto nas demais concentrações houve decréscimo na viabilidade celular quando comparados com o controle Hepes (n=6). Em preparações neuromusculares BCP, MERIV (5, 10 µg/mL) produziu um efeito facilitatório de 60 ± 15% e 46 ± 6%, respectivamente, seguido de bloqueio neuromuscular em 120 min de registro (n=6, $p<0.05$). De forma semelhante, a incubação dos músculos com Digoxina 52 nM ou Ouabaína 0,2 nM mimetizou a atividade de MERIV com aumento da amplitude de contração por 19 ± 4% e 27 ± 6%, e diminuição da contração muscular de 80 ± 4% e 91 ± 5%, respectivamente (n=5, $p<0.05$). MERIV também demonstrou atividade digitalic-like com inibição de 39 ± 3% da Na^+,K^+-ATPase (n=4, $p<0.05$). Em BPC, quando MERIV foi incubado 20 min antes da d-Tubocurarina 1,45 µM, houve um reforço do bloqueio neuromuscular, o qual foi completo em 80 min. Enquanto que em preparações BCP curarizadas, MERIV aumentou o tempo de bloqueio em 50 min, semelhante a ação de drogas anticolinesterásicas. Juntos, esses dados indicam que o extrato metanólico do veneno de *R. icterica* é capaz de interferir com a neurotransmissão provavelmente via inibição das enzimas acetilcolinesterase e Na^+-K^+- ATPase.

Palavras-chave: Veneno de sapo, junção neuromuscular, *biventer cervicis*, viabilidade celular, eletromiografia.

ABSTRACT

Animal poisons are sources of bioactive compounds with therapeutic applicability. Anurans through parotid glands produce a poisonous secretion rich in compounds of different chemical classes, that have a range of pharmacological activities of biotechnological interest. Toads of the species *Rhinella icterica* (Spix, 1824), belong to a group of poisonous animals present in the Pampa biome that still need to pharmacological and toxicological studies. Venom collection was made by milking toads obtained at Derrubadas region, Rio Grande do Sul state. The venom was previously treated by methanol extraction followed by lyophilization (thus called MERIV), before the biological assays. The venom neurobiology was evaluated on chicks neuromuscular junction by preparation *biventer cervicis* (BCP), and by function of mitochondrial dehydrogenases in hippocampal brain slices from mice. Incubation of MERIV (5, 10, 20 and 40 μg/mL) or digoxin (6.5, 13, 26 and 52 nM) with mice hippocampal brain slices induced a dose-dependent effect on cell viability. At low concentration MERIV (5 μg/mL) and digoxin (6.5 and 13 nM) induced a corresponding significative increases in cell viability, 36 ± 10%, 52 ± 7% and 57 ± 13% ($p<0.05$), respectively, while at higher concentrations there were a decrease in cell viability compared with control Hepes (n=6). In chicks neuromuscular preparation BCP, MERIV (5, 10 μg/mL) produced a facilitatory effect of 60 ± 15% and 46 ± 6%, respectively, followed by neuromuscular blockade in 120 min recordings (n=6, $p <0.05$). The incubation of BCP with digoxin (52 nM) or ouabain (0.2 nM) mimicked the venom activity by increasing the amplitude of the twitches by 19 ± 4% and 27 ± 6%, respectively, followed by a depression in muscle contraction recorded for 120 min (80 ± 4% and 91± 5%, $p<0.05$, respectively, n=5). MERIV also demonstrated digitalic-like activity inhibiting 39 ± 3% of Na^+,K^+-ATPase (n = 4, $p <0.05$). In BCP, when MERIV was incubated for 20 min before d-Tubocurarine (1.45 μM), there was a reinforcement of the neuromuscular blockade, wich was complete at 80 min. However, in preparations "curarizadas", incubated with d-Tubocurarine (1.45 μM) before MERIV, there was a increase in the blocking time at 50 min, similar to the action of acetylcholinesterase drugs. Altogether, these data indicate that the methanolic extract from *R. icterica* venom is able to interfere in neurotransmission, probably by inhibiting the enzymes acetylcholinesterase and Na^+,K^+- ATPase.

Key words: Toad venom, neuromuscular junction, biventer cervicis, cell viability, electromyography.

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE TABELAS

MANUSCRITO

LISTA DE ABREVIATURAS

ACh – Acetilcolina (Acetylcholine)

AChE – Acetilcolinesterase (Acetylcholinesterase)

AChRs – Receptores de acetilcolina (Acetylcholine Receptors)

BCP – Preparação *biventer cervicis*

Ca^{2+}-ATPase – Bomba de cálcio

ChAT – Colina acetil-transferase

ChT1 – Transportador de colina

d-Tc – d-Tubocurarina (d-Tubocurarine)

JNM – Junção neuromuscular

MERAV – Extrato metanólico do veneno de *Rhinella achavali*

MERIV – Extrato metanólico do veneno de *Rhinella icterica*

MERSV – Extrato metanólico do veneno de *Rhinella schneideri*

nAChRs – Receptores nicotínicos de acetilcolina

Na^{+},K^{+}-ATPase – Bomba de sódio e potássio

RS – Retículo sarcoplasmático

SNC – Sistema nervoso central

SNP – Sistema nervoso periférico

VAChT – Transportador vesicular de acetilcolina

APRESENTAÇÃO

No item INTRODUÇÃO, consta uma breve revisão da literatura sobre o tema abordado nesta dissertação.

A metodologia realizada e os resultados obtidos estão apresentados sob a forma de manuscrito em inglês para publicação em revista cientifica indexada e se encontram no item MANUSCRITO. No mesmo constam as informações sobre os Materiais e Métodos, a apresentação e descrição dos Resultados, a Discussão destes e as Referências Bibliográficas usadas na construção do texto.

Os itens CONSIDERAÇÕES FINAIS e PERSPECTIVAS, encontrados no final desta dissertação, apresentam interpretações e comentários gerais sobre os resultados deste trabalho.

A sessão REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação.

SUMÁRIO

1. INTRODUÇÃO

1.1. Venenos animais

Algumas espécies de animais produzem ou armazenam através da dieta, substâncias tóxicas denominadas biotoxinas. Esses compostos químicos são liberados como instrumentos no auxílio à captura de presas, bem como na defesa contra predadores (ABDEL-RAHMAN; AHMED; NABIL, 2010; ROSTELATO-FERREIRA et al., 2014; SAPORITO et al., 2012; SCIANI et al., 2013).

1.1.1. Venenos de anuros

Os sapos são caracterizados como animais venenosos, uma vez que a secreção venenosa à qual produzem em glândulas especializadas, não são passíveis de serem inoculadas, pela ausência de um aparato específico (dente oco, ferrão, aguilhão). Nesse sentido as glândulas secretoras estão distribuídas por toda a superfície corporal desses animais. As principais delas são as paratóides, glândulas granulosas, comumente agrupadas sobre a cabeça e pescoço; também há um grupo de glândulas menos desenvolvidas nas tíbias das patas posteriores, denominadas de paracnemis. As glândulas mucosas ocorrem em maior abundância e são responsáveis por produzir um muco viscoso sob a pele destes animais mantendo-os úmidos (DORNELLES; MARQUES; RENNER, 2010; FONSECA; MOREIRA; TSE, 2000).

As secreções das glândulas paratóides conferem proteção contra predadores, mas estas somente liberam o veneno ao serem pressionadas. Sendo assim, acidentes com sapos comumente ocorrem com animais domésticos, como cães e gatos, mas principalmente com cães criados em espaço confinado e que nunca tiveram contato com sapos, ao verem pela primeira vez acabam mordendo o animal, como brincadeira e ingerindo a secreção venenosa (BARRAVIERA, 1994). Os sintomas apresentados por estes envenenamentos variam de acordo com o tamanho do animal e a quantidade de veneno ingerido, podendo ocorrer hipersalivação, convulsões, vômitos, diarreias, arritimias, alucinações, perda da atividade motora, e, em alguns casos morte por parada cardio-respiratória (BARRAVIERA, 1994; SONNE et al., 2008). Casos de intoxicação em humanos são raros, os relatos são de mulheres romanas que tentavam matar os seus maridos utilizando veneno de sapos, sem muito sucesso, pois usaram apenas a água onde armazenavam esses animais (CHEN; KOVARIOVÁ, 1967).

Episódios de intoxicação também foram relatados com a solução tópica do “Chan su”, que é o veneno do sapo *Bufo bufo gargarizans* seco, utilizada como estimulante sexual, que ao ser exportada e por conter apenas a bula em chinês foi ingerida por algumas pessoas, as quais apresentaram sintomas de intoxicação por digitálicos (GOWDA; COHEN; KHAN, 2003). O veneno de sapo é principalmente cardiotóxico e determina envenenamento similar àquele causado por digitálicos (CHEN; KOVARIOVÁ, 1967; GOWDA; COHEN; KHAN, 2003).

1.1.2. Composição química dos venenos de anfíbios anuros

Nas secreções da pele e das glândulas dos anuros são encontrados diversas substâncias, como aminas, alcaloides, peptídeos, proteínas e esteroides, as quais apresentam uma série de atividades importantes para a farmacologia (SIANO et al., 2014). Os alcaloides agem como bloqueadores de canais iônicos neuronais do sistema nervoso central e periférico e do sistema muscular. Alguns estudos sugerem que a maioria dos alcaloides encontrados nos anfíbios é adquirida ou produzida por meio da dieta ou por bactérias do sistema digestivo, que são acumuladas nas glândulas e em outros órgãos (DALY; SPANDE; GARRAFFO, 2005; DALY, 1995; SAPORITO et al., 2004). Peptídeos e proteínas desempenham funções fisiológicas importantes como neuromoduladores e neurotransmissores, são também importantes ferramentas contra predadores e do sistema imunológico (CUNHA-FILHO et al., 2010). As neurotoxinas encontradas no veneno podem agir como bloqueadores de canais de cálcio, vasodilatadores, broncodilatadores, antitumorais, antidiabéticos e hepatoprotetores, além de poder agir como peptídeos opióides e neuropeptídios (ROMAN et al., 2012).

1.1.3. Atividade biológica dos venenos de anuros

As secreções produzidas através de glândulas encontradas na pele de anfíbios são ricas em componentes biologicamente ativos e com grande potencial biotecnológico. Há muito tempo, o veneno de sapo vem sendo utilizado para diversas funções por índios da América do Sul, que aplicam o veneno da pele de anuros da família Dendrobatidae em suas flechas para caçar ou se defender (CHEN; KOVARIOVÁ, 1967). No Japão o “Senso” e na China o “Chan su”, que são veneno de sapos da espécie *Bufo bufo gargarizans* (Cantor, 1842) seco, foi muito utilizado como expectorante, diurético, estimulante cardíaco, analgésico e antiinflamatório para uso em dores de dente, aftas e sinusites (CHEN; KOVARIOVÁ, 1967).

O veneno de sapos atua quando entra em contato com mucosas e os sintomas são imediatos. O veneno é composto por uma mistura de substâncias quimicamente ativas, em

que se destacam as que possuem mecanismo de ação semelhantes a adrenalina, digitálicas e neurotoxinas.

Ensaios *in vitro* tem demonstrado que bufalin, um composto bloqueador neuromuscular isolado do veneno de *Bufo bufo gargarizans*, além de outros bufadienolídeos e cinobufagin, são os maiores componentes presentes no Chan su, os quais induzem apoptose e/ou inibição da progressão do ciclo celular em uma variedade de células neoplásicas, incluindo as de leucemia, câncer de próstata, câncer de estômago e câncer de fígado (CUNHA-FILHO et al., 2010; YIN et al., 2012) . Além disso, em um tratamento experimental com Huachansu, veneno da pele de sapo *Bufo bufo gargarizans*, houve regressão de 20% do câncer de fígado em um dos pacientes (CHOI; HONG, 2012; DENG et al., 2014; MORENO Y BANULS et al., 2013; TAKAI et al., 2012; YIN et al., 2012).

Bufadienolídeos isolados de *Rhinella jimi*, têm demonstrado atividade contra Leishmania (L.) e Trypanosoma cruzi (TEMPONE et al., 2008). Esses compostos também possuem atividade anestésica, antiviral, antibacteriana e inseticida, além de inibirem a Na^+,K^+ ATPase; também foi relatada uma atividade hipoglicemiante nesses compostos (YANG et al., 2015).

Do ponto de vista farmacológico os bufadienolídeos exibem atividade semelhante aos hormônios endógenos relacionados com o bloqueio da Na^+,K^+-ATPase, como antiangiogênicos, anti-hipertensivos, imunossupressores, antiendometriose, desencadeando uma ação ação inotrópica positiva (KAMBOJ; RATHOUR; KAUR, 2013). Nesse sentido, a cardiotoxicidade induzida por venenos de sapo em vertebrados, tem seu mecanismo de ação bem descrito, relacionado à presença de glicosídeos cardíacos (GOWDA; COHEN; KHAN, 2003; KUO et al., 2007; KWAN; PAIUSCO; KOHL, 1992; RADFORD et al., 1986; ROHRER et al., 1982).Yoshida e Sakai publicaram estudos nos quais demonstraram os efeitos inibitórios de bufalin sobre a junção neuromuscular de murinos (SEIICHIRO YOSHIDA AND TAKESHI SAKAI, 1973, 1974). Manika e Gomes descreveram que o fator letal (TSE-LF), isolado e purificado da pele de *Bufo melanostictus* (Schneider, 1799), tem induzido ação neurotóxica em preparação *biventer cervicis* de pintainho (MANIKA DAS; GOMES, 2001). Recentemente, Rostelato e colaboradores, demonstraram um efeito pré-sináptico importante no veneno de sapo *Rhinella schneideri* (Werner, 1894), usando preparações neuromusculares de aves e mamíferos.

O veneno de anfíbios da família Bufonidae é extremamente tóxico e sua biologia é pouco conhecida. Além disso, venenos animais são muito importantes na biofarmacologia e

possuem elevado potencial biotecnológico. O conhecimento sobre esses venenos pode auxiliar na síntese de substâncias quimicamente ativas com possibilidade de uso terapêutico (BARRAVIERA, 1994).

1.1.4. A espécie *Rhinella icterica*

Os sapos são vertebrados pertencentes ao filo Chordata, classe Amphibia e a ordem Anura. Atualmente são conhecidas aproximadamente 6.580 espécies que compõe a ordem Anura e estão distribuídas em 54 famílias, presentes em todo globo, exceto na Antártica e algumas ilhas oceânicas (FROST et al. 2015). De acordo com a Sociedade Brasileira de Herpetologia (SEGALLA et al., 2014), até a presente data foram reconhecidas 1026 espécies de anfíbios ocorrentes no Brasil, sendo 988 espécies da ordem Anura, ocupando assim, a primeira colocação na relação de países com maior riqueza de espécies da ordem Anura.

A família Bufonidae está representada por 49 gêneros e 586 espécies, apresentando ampla distribuição geográfica, ocorrendo em quase todos os continentes, com exceção da Austrália, Nova Guiné e Madagascar (FROST et al. 2015).

A espécie *R. icterica* foi descrita em 1824 por Spix inicialmente como *Bufo icterica*, posteriormente, denominada de *Chaunus icterica*, e atualmente *Rhinella icterica* (Figura 1).

Classificação científica:

- Reino: Animalia
- Filo: Chordata
- Classe: Amphibia
- Ordem: Anura
- Família Bufonidae
- Gênero: Rhinella
- Espécie: *Rhinella icterica*

Figura 1: *R. icterica* macho (a esquerda) e fêmea (a direita).
Fonte: http://br.herpeto.org/anfibios/anura/rhinella-icterica/

É um animal nativo da América do Sul, mais especificamente no Brasil, Argentina e Paraguai (BALDO et al, 2010). São anfíbios de grande porte (macho 98-130 mm, fêmea 110-165 mm) possuindo membros curtos, coloração que varia de castanho-claro a escuro e pele áspera com região dorsal bastante rugosa, devido à presença de glândulas cutâneas (LEMA; MARTINS, 2011).

As glândulas cutâneas se espalham pelo corpo do animal e produzem substâncias tóxicas conhecidas como batracotoxinas, que como em sapos de outras espécies, são usadas como forma de defesa contra predadores e microorganismos patogênicos (DALY, 1995). A liberação do veneno das glândulas ocorre em situações de estresse ou contrição mecânica e é provocada por regulação nervosa.

A intoxicação pelos venenos de sapos do gênero Rhinella geralmente ocorre por alterações do sistema cardiovascular, do sistema nervoso periférico e central de mamíferos, sendo possivelmente associada à grande variedade de proteínas tóxicas (Figura 2).

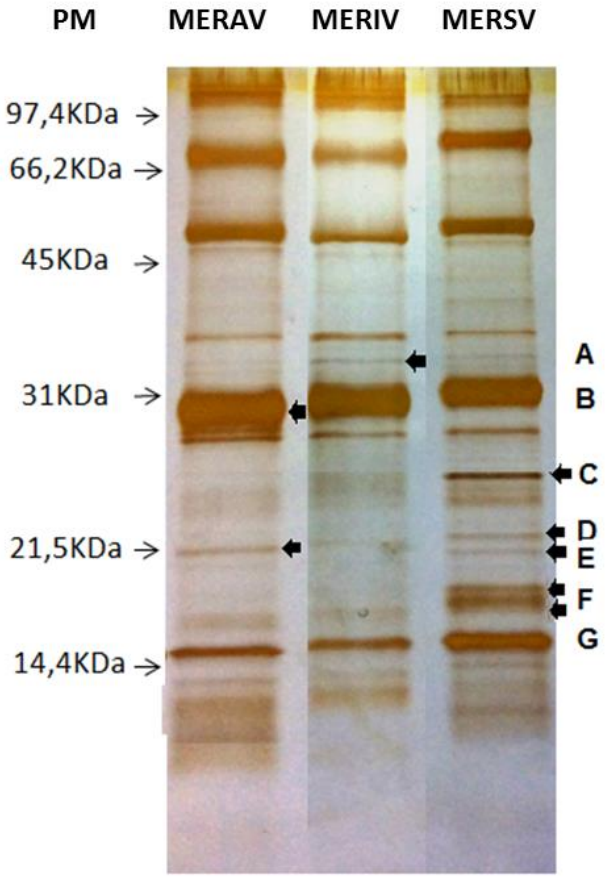


Figura 2: SDS Page dos venenos de *R. achavali* (MERAV), *R. icterica* (MERIV) e *R. schneideri* (MERSV). As setas ao lado esquerdo indicam os padrões de peso molecular (PM): fosfolipase B (97,4 kDa), albumina sérica bovina (66,2 kDa), ovoalbumina (45 kDa), anidrase carbônica (31 kDa), inibidor de tripsina(21,5 kDa) e lisozima (14,4 kDa). As setas ao lado direito indicam as bandas de A a G que representam as diferenças de proteínas presentes nos venenos. Fonte: LANETOX – Laboratório de Neurobiologia e Toxinologia.

Assim, as substâncias presentes no veneno podem ser divididas basicamente em dois grupos: compostos básicos (aminas biogênicas) e derivados esteroides (SAKATE; LUCAS DE OLIVEIRA, 2000). As aminas biogênicas incluem adrenalina, noradrenalina, bufoteninas, dihidrobufoteninas e bufotioninas. As bufoteninas, dihidrobufoteninas e bufotioninas são responsáveis pelos efeitos alucinógenos no sistema nervoso central e aumentam a liberação de neurotransmissores no sistema nervoso periférico (ROSTELATO-FERREIRA et al., 2011; SONNE et al., 2008). Os derivados esteroides incluem colesterol, ergosterol, bufotoxinas e bufadienolídeos. Os bufodienolídeos e bufotoxinas são

responsáveis pela ação digitálica induzida pelos venenos de sapos (CUNHA-FILHO et al., 2010). Dessa forma, o efeito digitálico é responsável pela inibição da Na^+,K^+-ATPase nas células cardíacas, aumentando a concentração intracelular de sódio e, consequentemente, inibindo a extrusão do cálcio (RANG et al., 2005). O aumento da concentração de cálcio nos miócitos cardíacos, produz um aumento da força de contração cardíaca (inotropismo positivo) e reduz a frequência dos batimentos cardíacos (cronotropismo negativo) por uma ação vagal (CHEN et al., 2006). O bloqueio da bomba de Na^+,K^+-ATPase no sistema nervoso induz um aumento da liberação de neurotransmissores, um efeito ainda pouco estudado (ARNAIZ; BERSIER, 2014).

1.2. Neurotransmissão

A transmissão de mensagens entre duas células se dá através de sinapses, uma junção especializada em que um terminal axonal faz contato com outro neurônio ou tipo de célula. O sentido normal do fluxo de informação é do terminal axonal para o neurônio-alvo; desse modo, o terminal axonal é o pré-sináptico, enquanto o neurônio-alvo é o pós-sináptico. A transmissão sináptica envolve a conversão do impulso nervoso, de natureza elétrica, em uma mensagem química carreada por substâncias neuromediadoras, e depois novamente em impulsos elétricos já na célula pós-sináptica (BEAR; CONNORS; PARADISO, 2002; LENT, 2010; SILVERTHORN, 2010).

Há dois tipos básicos de sinapses: as químicas e as elétricas. As sinapses elétricas são chamadas de junções comunicantes, possuem estrutura mais simples, transferem correntes iônicas e até mesmo pequenas moléculas entre células acopladas. As sinapses químicas podem modificar as mensagens que transmitem de acordo com inúmeras circunstâncias. Sua estrutura é especializada no armazenamento de substâncias neurotransmissoras e neuromoduladoras que, quando liberadas na fenda sináptica, provocam alterações de potencial elétrico na membrana pós-sináptica, que poderão influenciar o disparo de potenciais de ação do neurônio pós-sináptico. O resultado da interação dos efeitos excitatórios e inibitórios de cada uma das sinapses sobre o potencial da membrana do neurônio pós-sináptico irá definir a mensagem transportada pelo axônio do segundo neurônio, em direção a outras células (BEAR; CONNORS; PARADISO, 2002; LENT, 2010).

1.2.1. Junção Neuromuscular (JNM)

A junção neuromuscular (JNM), também chamada de placa motora, é uma sinapse química colinérgica com a função de transferir impulsos de uma terminação nervosa para uma fibra muscular e, assim, desencadear a contração muscular, por meio da ação do neurotransmissor acetilcolina (ACh) . A JNM é formada por três elementos estruturais: a região pré-sináptica, onde se encontra a terminação nervosa; a região pós-sináptica, célula muscular; e a fenda sináptica, fenda entre 20 e 30 nm que separa as membranas das células pré e pós-sináptica (HUGHES; KUSNER; KAMINSKI, 2006) (Figura 3).

Os neurotransmissores são liberados para a fenda sináptica pelos botões sinápticos, formado por expansões das extremidades do axônio que perde sua bainha de mielina quando está próximo da placa motora. Cada botão sináptico sobrepõe depressões presentes na superfície da fibra muscular, chamadas de fendas subneurais, sendo este um local de alta densidade de receptores de ACh (AChR). Além de que, em cada botão sináptico está presente a maquinaria necessária para liberar o neurotransmissor: vesículas sinápticas onde a ACh é armazenada; complexo de acoplamento, complexo proteico de exocitose das vesículas; zona ativa, sítio de liberação do neurotransmissor; e canais de Ca^{2+} voltagem-dependentes, os quais permitem a entrada de Ca^{2+} na terminação nervosa a cada potencial de ação. O Ca^{2+} é responsável por desencadear a fusão das vesículas sinápticas com a zona ativa, liberando seu conteúdo para a fenda sináptica (BEAR; CONNORS; PARADISO, 2002; HUGHES; KUSNER; KAMINSKI, 2006; KATZ, 1966; LENT, 2010). O resultado da reação entre o neurotransmissor e o seu receptor, como acontece em todas as sinapses excitatórias, é a abertura seletiva de canais de Na^{+} e K^{+}, e a ocorrência de um potencial pós-sináptico despolarizante (BEAR; CONNORS; PARADISO, 2002; LENT, 2010). Esta reação pode ser impedida caso estejam presentes as toxinas d-Tubocurarina e/ou α-bungarotoxina pois estas são capazes de inibir a transmissão neuromuscular por bloquearem os receptores colinérgicos nicotínicos da placa motora (MELLA; AMBIEL; PRADO, 2000).

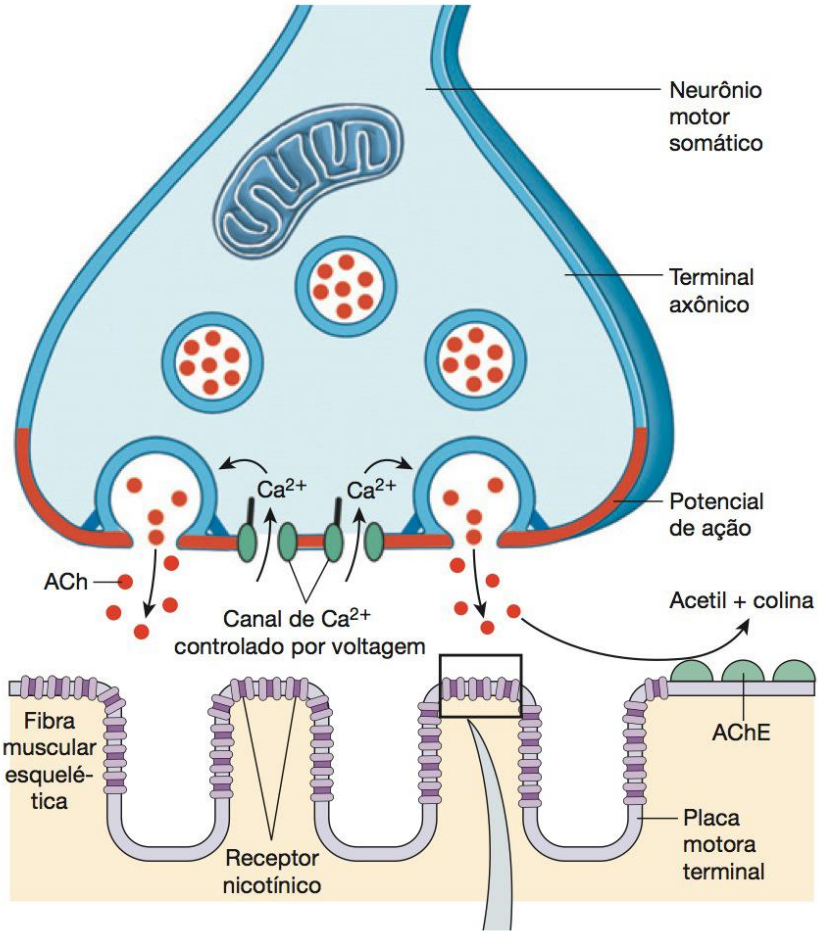


Figura 3: Eventos fisiológicos na junção neuromuscular. A chegada de potenciais de ação no terminal do axônio abre canais de Ca^{2+} dependentes de voltagem. O Ca^{2+} difunde-se para dentro da célula a favor do gradiente eletroquímico, desencadeando a liberação de ACh contida nas vesículas sinápticas. A ACh difunde-se através da fenda sináptica e combina-se com receptores nicotínicos (nAChRs) na membrana do músculo esquelético. Fonte: (SILVERTHORN, 2010).

A ACh é sintetizada no terminal pré-sináptico pela enzima colina acetiltransferase (ChAT) a partir da colina e do acetil-CoA. Após, a acetilcolina é armazenada no interior das vesículas por seu transportador vesicular, VAChT. Durante a neurotransmissão, as vesículas sinápticas, passam por um ciclo nos terminais nervosos, onde primeiramente os neurotransmissores são transportados para o interior das vesículas sinápticas, que se agrupam nas adjacências da zona ativa, após haverá ancoramento e estas estarão aptas para a fusão e liberação de seu conteúdo na fenda sináptica (ARISI; NEDER; MOREIRA, 2001). Após a exocitose e ativação dos receptores nicotínicos, a ACh é degradada pela enzima

acetilcolinesterase (AChE) presente na fenda sináptica, gerando colina e acetato, o que a torna inativa para os AChRs. A colina é recaptada para o interior do terminal por meio de seu transportador de membrana (CHT1) e será utilizada novamente para a síntese de acetilcolina. A AChE é responsável por evitar a exposição ininterrupta de ACh na junção neuromuscular o que pode causar dessensibilização dos receptores e bloqueio da transmissão neuromuscular. Em pequenas quantidades, inibidores da AChE, como a Neostigmina, podem reforçar as transmissões neuromusculares prolongando a ação da ACh liberada (BEAR; CONNORS; PARADISO, 2002; BUAINAIN; MOURA; OLIVEIRA, 2000; SOUSA et al., 2006).

1.2.2. Bomba de Na^+,K^+-ATPase

A Na^+,K^+-ATPase (EC 3.6.1.37) é o maior complexo de proteína na família de bombas de cátions do tipo P. É uma enzima de membrana integral que transporta 3 íons Na^+ para fora da célula e 2 íons K^+ para dentro da célula, contra a correspondente gradiente de concentração das células. O gradiente produzido por esta enzima é necessário para a manutenção de funções fisiológicas, tais como a proliferação celular, regulação do volume celular, potenciais de membrana, crucial para tecidos excitáveis como músculos e nervos, e de transporte ativo secundário de outros solutos (HORVAT et al., 2006; JORGENSEN; HÅKANSSON; KARLISH, 2003). O gradiente eletroquímico favorável para a entrada de íons Na^+ fornece a energia necessária para que o transporte para fora da célula de íons Ca^{2+}, contra o seu gradiente eletroquímico. Assim, a inibição da Na^+,K^+-ATPase interfere indiretamente na atividade do trocador Na^+-Ca^{2+}, causando a reversão de seu funcionamento com influxo de Ca^{2+} e o efluxo de Na^+ (PALTY; SEKLER, 2012) (Figura 4). Por consequência, mais Ca^{2+} é acumulado no Retículo Sarcoplamático (RS) por ação de sua Ca^{2+}-ATPase e disponibilizado durante as contrações subsequentes, o que favorece o aumento da força contrátil (LI; XIE, 2009).

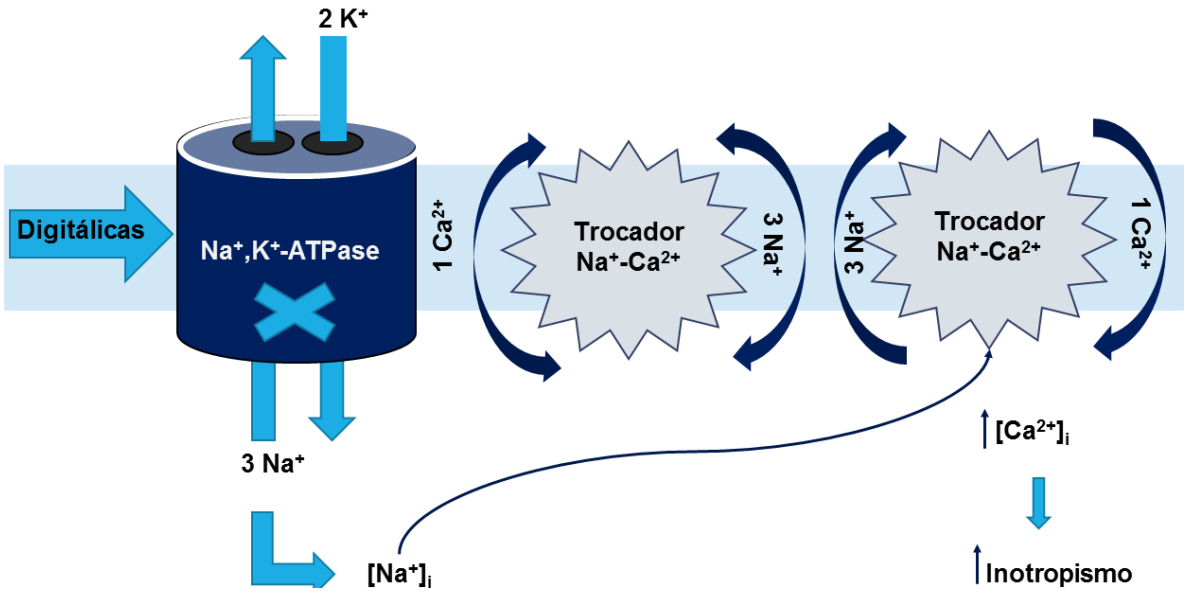


Figura 4: Representação do mecanismo de ação das digitálicas. A Na^+,K^+-ATPase controla a concentração citoplasmática de Na^+ em repouso o que determina a concentração de Ca^{2+} através do trocador Na^+/Ca^{2+}. A inibição da Na^+,K^+-ATPase causa a reversão do funcionamento do trocador Na^+-Ca^{2+}, com influxo de Ca^{2+} e o efluxo de Na^+. Estes eventos influenciam a atividade da Ca^{2+}-ATPase e a atividade do canal de Ca^{2+}. Assim, mais Ca^{2+} é acumulado no Retículo Sarcoplamático (RS) por ação de sua Ca^{2+}-ATPase e disponibilizado durante as contrações subsequentes. Em conjunto, todos esses eventos são responsáveis por controlar o nível intracelular de Ca^{2+} e influenciam a contratilidade muscular, cardíaca e a excitabilidade neuronal, o que favorece o aumento da força contrátil. (HORVAT et al., 2006; LI; XIE, 2009). Fonte: Autor.

Esta enzima também é conhecida como ouabaina-sensível devido ao efeito farmacológico de ser especificamente inibida por esteroides cardiotônicos, como um receptor de ouabaína (HORVAT et al., 2006).

Os esteroides cardiotônicos, assim chamados por possuírem ação inotrópica positiva sobre o coração, são inibidores da Na^+,K^+-ATPase e sua estrutura química básica está em torno de um núcleo esteroidal. São encontrados tanto em plantas quanto em animais. A digoxina e a ouabaína são extraídas das folhas secas da plantas *Digitalis purpúrea* L. e *Strophantus gratus* (Wall. & Hook.) Baill, são esteroides cardiotônicos utilizados no tratamento de insuficiência cardíaca congestiva, quando o coração não tem força suficiente para a contração muscular (SHAMAGIAN et al., 2005). Os sapos contém esteroides nas secreções de suas glândulas paratoides, sendo os bufadienolídeos presentes no veneno responsáveis pelo envenenamento de pequenos animais, causando convulsões e parada

cardíaca (CUNHA-FILHO et al., 2010; TEMPONE et al., 2008). A ação cardiotóxica do veneno é semelhante à intoxicação por digitálicas e apresenta o mesmo mecanismo que promove inotropismo positivo decorrente da inibição da Na^+,K^+-ATPase (DE ROBERTIS; HIB, 2006; GOWDA; COHEN; KHAN, 2003).

1.2.3. Viabilidade Celular

A viabilidade celular é caracterizada pela capacidade que uma célula possui de realizar determinadas funções, como metabolismo, crescimento e reprodução. A divisão celular garante a reparação tissular ou celular após uma lesão que tenha causado uma perda de células (RANG et al., 2005). Porém, mesmo no estado adulto, as células morrem e é imprescindível sua substituição (RANG et al., 2005). A morte celular pode ocorrer de duas formas distintas, por necrose ou apoptose. A apoptose funciona como um sistema controlador da homeostase através da eliminação não inflamatória de células irreversivelmente lesadas ou desnecessárias (YIN et al., 2012). A mitocôndria pode desencadear a apoptose ao liberar para o citoplasma proteínas pró-apoptóticas, como o citocromo c e fator indutor de apoptose (DENG et al., 2014).

A membrana celular é a parte mais externa da célula o que a torna um sítio receptor ideal para substâncias químicas, que podem ser toxinas, drogas e hormônios. Estas, por sua vez, podem interagir com as proteínas presentes na membrana ou com seus componentes lipídicos, alterar suas funções de transporte e a integridade da célula como um todo. Os receptores apresentam sítios para ligação de moléculas, estas atuam como moduladores destes receptores (RANG et al., 2005). Estudos sugerem que a enzima Na^+,K^+-ATPase possui além de seu papel regulatório na homeostasia iônica, um papel importante na transdução de sinal e na ativação da transcrição gênica, modulando na presença de ouabaína o crescimento e migração celular e a apoptose (BAGROV; SHAPIRO; FEDOROVA, 2009; DANIEL et al., 2010; DVELA et al., 2012; KOTOVA et al., 2006). Além disso, os estudos sugerem a relevância fisiológica da ouabaína e glicosídeos cardiotônicos em regular a viabilidade celular quando em concentrações da mesma amplitude de seus níveis circulantes (CHEN et al., 2006; DVELA et al., 2012)

2. JUSTIFICATIVA

O Brasil é o país com maior biodiversidade de anfíbios anuros e o estado do Rio Grande do Sul contém cerca de 10% dessa diversidade, com 98 espécies de anuros descritos até o momento. Porém muitas dessas espécies ainda carecem de estudos farmacológicos e toxicológicos. Apesar do óbvio interesse ecológico e clínico no estudo dos sapos, a presença de substâncias biologicamente ativas no veneno, aumenta o interesse no estudo desses animais como fonte de compostos com aplicabilidade biotecnológica (Monti e Cardello, 1994).

Considerando a grande biodiversidade de anfíbios ainda não catalogados e que toxicologicamente ainda não foram explorados, e que as toxinas produzidas por eles podem possuir propriedades terapêuticas para vertebrados, há interesse em investigar os seus mecanismos de ação fisiológica, e assim, aumentar o conhecimento sobre anfíbios anuros e posteriormente conduzir ao desenvolvimento de fármacos que possam ser adicionados a coleção de compostos biologicamente ativos.

3. OBJETIVOS

3.1. Objetivo Geral

Caracterizar a atividade neurobiológica do veneno do sapo *R. icterica* em vertebrados.

3.2. Objetivos específicos

- Caracterizar por meio de teste eletromiográfico o efeito do veneno de *R. icterica in vitro* em preparação neuromuscular *biventer cervicis* de pintainhos (*Gallus gallus domesticus*).
- Comparar os efeitos do veneno de *R. icterica* com a ação de drogas inibidoras da Na^+,K^+-ATPase em preparação neuromuscular de pintainho.
- Verificar o efeito anticolinesterásico do veneno de *R. icterica* em preparação neuromuscular de pintainho.
- Avaliar a viabilidade celular do veneno de *R. icterica* em fatias de hipocampo de camundongos (*Mus musculus*).
- Comparar os efeitos do veneno de *R. icterica* com a ação de drogas inibidoras da bomba de Na^+/K^+ em fatias hipocampais de camundongos.
- Avaliar a atividade inibidora da Na^+,K^+-ATPase do veneno de *R. icterica* em tecido cardíaco de ratos (*Rattus norvegicus*).

4. MANUSCRITO

Todos os Resultados, bem como os itens Materiais e Métodos, Discussão e Referências Bibliográficas que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. Este manuscrito está disposto na forma na qual deverá ser submetido para o periódico **Chemico Biological Interactions** (ISSN: 0009-2797) e será intitulado como: ***In Vitro* Neurobiology of *Rhinella icterica* (Spix, 1824) Toad Venom in Chicks and Mammalian Preparations.**

In vitro Neurobiology of *Rhinella icterica* (Spix, 1824) Toad Venom in Chicks and Mammalian Preparations

Raquel Soares Oliveira[a], Allan Pinto Leal[a], Carlos Gabriel Moreira de Almeida[a], Douglas Silva dos Santos[b], Leandro Homrich Lorentz[a], Cleci Menezes Moreira[c], Tiago Gomes dos Santos[a], Cháristo André Dal Belo[a,b] and Lúcia Vinadé[a*]

[a]*LANETOX, Universidade Federal do Pampa (UNIPAMPA),Av. Antônio Trilha 1847, 97300-000, São Gabriel, RS, Brazil*
[b]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, (PPGBTox), Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000,97105-900, Santa Maria, RS, Brazil*

*Corresponding authors:

Dra. Lúcia Vinadé
CIPBiotec, campus SãoGabriel, Universidade Federal do Pampa
Av.Antônio Trilha,1847
97300000 São Gabriel, Brazil
Tel./fax: + 55 55 3237-0850.
luciavinade@unipampa.edu.br

Dr. Cháristo André Dal Belo
CIPBiotec, campus São Gabriel, Universidade Federal do Pampa
Av. Antônio Trilha,1847
97300000 São Gabriel, Brazil
Tel./fax: + 55 55 3237-0850.
charistonbelo@unipampa.edu.br

Abstract

The neurobiology of *Rhinella icterica* toad venom (MERIV) was evaluated on chick neuromuscular junctions and in mice hippocampal slices. The assessment of *biventer cervicis* acetylcholinesterase activity in presence of MERIV showed a significative inhibition of this enzyme. Incubation of MERIV (5 μg/mL) or digoxin (6.5 and 13 nM) at mice hippocampal brain slices increased significantly the cell viability, when compared to Hepes saline. At chick nerve muscle preparations MERIV (5, 10 μg/mL) produced a dose-independent transitory increase of muscle twitch tension, followed by an irreversible neuromuscular blockade in 120 min recordings. At this set of experiments, the incubation of muscles with digoxin (52 nM) or ouabain (0.2 nM) mimicked the venom activity by increasing the amplitude of the twitches followed by a progressive depression of the muscle strength in 120 min recordings ($n=5$, $p<0.05$). MERIV also demonstrated a digitalic-like activity by inhibiting significantly the Na^+,K^+-ATPase. At chick neuromuscular junctions, the incubation of MERIV partially prevented the curare neuromuscular blockade. Altogether, these data indicate that the methanolic extract from *R. icterica* venom is able to interfere with peripheral and central neurotransmission, probably by inhibiting the activities of the enzymes acetylcholinesterase and the Na^+,K^+-ATPase.

Key words: Toad venoms, vertebrates, central neuroprotection, neuromuscular blockade, AChE, Na^+,K^+-ATPase.

1. INTRODUCTION

Animal venoms are rich sources of bioactive compounds with therapeutic applicability. Venomous animals generally synthesize chemical defenses against predators and parasites (HEUS et al., 2014; SAPORITO et al., 2012). Toads are animals which have cutaneous glands around the body that produce a poisonous secretion with biotechnological interest (CUNHA-FILHO et al., 2010; SCIANI et al., 2013). Studies have identified in the skin and cutaneous glands of amphibians secretions that present peptides, alkaloids, steroids, biogenic amines and proteins (CUNHA-FILHO et al., 2010; DALY, 1995; SCIANI et al., 2013; SIANO et al., 2014). Specifically in the secretions of toad venoms there are molecules generally associated to the maintenance of humidity and cutaneous respiration, thermoregulation, chemical defense against predators and microorganisms (CUNHA-FILHO et al., 2010; ROSTELATO-FERREIRA et al., 2014; SAKATE; LUCAS DE OLIVEIRA, 2000; SCIANI et al., 2013).

Among the pharmacological activity induced by toad venoms there are a number of works describing effects related to peripheral neurotoxicity. In this regard, Yoshida and Sakai (SEIICHIRO YOSHIDA AND TAKESHI SAKAI, 1973, 1974) have published a couple of articles showing the effects of a neuromuscular blocker compound named bufalin, that was isolated from the venom of *Bufo gargarizans* (Cantor, 1842). Manika and Gomes (MANIKA DAS; GOMES, 2001) have described that the lethal factor (TSE-LF) isolated and purified from *Bufo melanostictus* (Schneider,1799) skin has induced neurotoxic action on chick *biventer cervicis*. Recently, Rostelato and cols (ROSTELATO-FERREIRA et al., 2011, 2014) have demonstrated an interesting presynaptic effect on *Rhinella schneideri* (Werner, 1894) toad venom, using avian and mammalian neuromuscular preparations. The cardiotoxicity induced by toad venoms in vertebrates are another well described biological activity and is related to the presence of cardiac glycosides (GOWDA; COHEN; KHAN, 2003; KUO et al., 2007; KWAN; PAIUSCO; KOHL, 1992; RADFORD et al., 1986; ROHRER et al., 1982).

R. icterica, the "cururu toad", is a large anuran (up to 140 mm) of the Bufonidae family, native to South America, with a broad geographic distribution, occurring in the southeastern and southern Brazil, Paraguay, Uruguay and Argentine(COLOMBO et al., 2008). However, as far as our knowledge, none preliminary pharmacological study has been undertaken with *R. icterica* venom at vertebrate nervous system. In this work, we sought to investigate the biological activity of *R. icterica* venom at mammalian central nervous system and chicks' neuromuscular junctions. The rationale of this work was determining the mechanism of action of *R. icterica* venom by using biochemical and neurophysiological *in vitro* preparations.

2. MATERIAL AND METHODS

2.1. Reagents and venom

All chemicals and reagents used were of the highest purity and were obtained from Sigma-Aldrich, Merck or BioRad. The animals were collected by Prof. Tiago Gomes dos Santos, with authorization of the System Authorization and Information on Biodiversity (SISBIO) Collector License No: 24041-2. After extraction of the venom, animals were tumbled in collection.Venom collection was made by milking toads obtained at Derrubadas region located at the northwest of Rio Grande do Sul. *R. icterica* venom (MERIV) was previously treated by methanol extraction followed by lyophilization according Rostelato (2011), resulting in a methanolic extract used in all biological assays.

2.2. Animals

Adult Swiss mice and male Wistar rats were supplied by the animal facilities from the Federal University of Santa Maria (UFSM, Brazil). Hyline chicks (1 - 10 days) were supplied by local seller (Agropecuaria Sinuelo, São Gabriel, RS, Brazil). The animals were housed with water and food *ad libitum* in controlled temperature and lighting (± 25°C and 12-hour cycles light / dark). Adult cockroaches (*Nauphoeta cinerea*, 3-4 month after adult molt) were used to assess the potential modulatory activity induced by the venom on acetylcholinesterase activity. The animals were reared at laboratory conditions with controlled temperature (22-25ºC) on a 12h:12h L:D cycle. All cockroaches were provided with water and dog chow *ad libitum*. This work was approved by the Institutional Committee for Ethics in Animal Use (CEUA/ UNIPAMPA, Protocol nº 037/2012).

2.3. Na^+, K^+-ATPase activity

The potential of MERIV to influence the Na^+, K^+-ATPase activity was investigated in rat´s hearts as described elsewhere Stefanon and collaborators (STEFANON et al., 2009). Briefly, ventricular tissue was homogenized in a solution containing 20 mM Tris–HCl and 1 mM EDTA, pH 7.5. The homogenized tissue was centrifuged at 8.800 rpm for 20 min and the precipitate was discarded. The supernatant was then centrifuged at 10.000 rpm for 60 min. The precipitate was resuspended in 20 mM Tris–HCl and 1 mM EDTA, pH 7.5 in a final volume of 400 μL. Na^+, K^+-ATPase activity was assayed by measuring Pi liberation from 3 mM ATP in the presence of 125 mM NaCl, 3 mM MgCl2, 20 mM KCl, and 50 mM Tris–HCl (pH 7.5). The enzyme was pre-incubated for 5 min at 37ºC, then the reaction was initiated by adding ATP. The incubation times and protein concentration were chosen to ensure the linearity of the reaction. The reaction was quenched by the addition of 200 μL of 10% trichloroacetic acid. Control reactions, where the enzyme was added following addition of trichloroacetic acid, were used to correct for any non-enzymatic hydrolysis of the substrate. All samples were examined in duplicate. The specific activity was reported as nmol Pi released per min per mg of protein, unless otherwise stated. The specific activity of the enzyme was determined in the presence and absence of 5 μg/mL MERIV.

2.4. Assay for Acetylcholinesterase activity (AChE)

The *in vitro* inhibition of AChE was evaluated according to ELLMAN *et al.*(ELLMAN et al., 1961) using both cockroach brain (STÜRMER et al., 2014) and in biventer cervicis muscle homogenates. Briefly, after electromyographic protocols, the biventer cervicis muscles were homogenized in phosphate-buffer saline (pH 7.0) and centrifuged at 1000xg for 5 min at 4ºC. The supernatant was used for determination of AChE activity in spectrophotometer at 412 nm. The assays using cockroaches were performed after injection of different doses of MERIV at the third abdominal region, by means of Hamilton syringe (final

volume 20µL). After six hours following the treatments the animals had their heads removed, the brains (at least six animals) were homogenized in phosphate-saline buffer (pH 7.0) and centrifuged at 1000xg for 3 min at 4ºC. The supernatant was used for determination of AChE following the same protocol used for chick muscles.

2.5. Hippocampal Slices Preparation and MTT assay

The MTT colorimetric assay was performed on hippocampal slices in the presence or absence of MERIV. Mice were decapitated, the brains removed immediately to hippocampus dissection on ice, and the hippocampus dissected on ice and humidified in cold HEPES-saline buffer gassed with O_2 (124mM NaCl, 4mM KCl, 1.2mM $MgSO_4$,12mM glucose, 1mM $CaCl_2$, and 25mM HEPES pH 7.4). Hippocampal slices were obtained according to Vinadé and Rodnight (VINADÉ; RODNIGHT, 1996), modified by Dal Belo and collaborators (DAL BELO et al., 2013): a Mcilwain tissue chopper was used to obtain the slices (0.4µm) that were separated and preincubated at 37ºC for 30 min in micro well plates filled with HEPES saline (200 µL/slice). Then, the medium was replaced (200 µL/slice) for control condition and treatments with MERIV (5, 10, 20 and 40 µg/mL) and incubated for 60 min (37ºC). Immediately after incubation, slices were assayed for a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test (0.05% in HEPES-saline) for 30 min (37°C). The MTT was converted into a purple formazan product after cleavage of the tetrazolium ring by dehydrogenases. Formazan was dissolved by the addition of 100% DMSO, resulting in a colored compound whose optical density (λ = 490 nm) was measured in an ELISA reader equipment.

2.6. Chick biventer cervicis preparation

The animals were sacrificed under anesthesia using halothane. The muscle *biventer cervicis* was isolated and assembled according to the method described by Ginsborg and Warriner (GINSBORG; WARRINER, 1960). The muscles were placed on an isolated organ bath (AVS Projetos, mod IOB, São Paulo, Brazil) with capacity of 5 mL containing Krebs solution (in mM: 136 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11 Glucose, 23.8 $NaHCO_3$, pH 7.5). The solution was keept aerated with carbogen (95% O_2 and 5% CO_2) at 37°C. The preparation was subjected at 0.5 g/cm tension. Electrical stimulation was delivered by bipolar electrodes positioned in the region between the tendon and muscle in order to establish a field stimulation. Supraximal stimuli were applied to the muscle (0.1 Hz frequency and 0.2 ms duration) from a stimulator (AVS Projetos, mod. 100-C4). Muscle contractions resulting from maximal electrical stimulation and contractions in response to the addition of KCl (13.4 mM) and ACh (120 mM) were recorded for 120 min on a physiograph via an 1g isometric transducer (AVS Projetos, mod IOB, São Paulo, Brazil). To observe the effect of MERIV or pharmacological treatments on muscle contractions in response and following the application of KCl and ACh, treatments were added to the preparation after 20 min preliminary stabilization period. After de addition of the treatments the recordings were done during 120 min after that KCL and ACh were added again in order to compare the contraction responses with control Krebs.

2.7. Statistical analysis

Each experimental protocol was repeated 3-8 times and the results were expressed as the mean ± SEM. Differences were validated by ANOVA and Tukey test as *post hoc* with $p<0.05$ indicating significance. All data analyses were performed using SAS Enterprise Guide 4.3 and GraphPad Prism.

3. RESULTS

3.1.1. Na^+,K^+-ATPase activity

The analysis of the Na^+,K^+-ATPase in rat's heart activity in presence of MERIV (5µg/ml) showed that this compound inhibited significatively the electrogenic pump in 39±3% (n=4, $p<0.05$) (Fig. 2).

3.1.2. Acetylcholinesterase (AChE) activity of MERIV

The analysis of AChE activity on cockroach brain homogenates of animals injected with different concentrations of MERIV (5, 10, 20 and 40 µg/g) or neostigmine (0.5µM) revealed a significant enzyme inhibition, compared to the control (insects injected with saline only). When MERIV was incubated (5 µg/g animal weight) the AChE activity decreased to 21 ± 2% (n=3; $p<0.05$). The percentage of AChE activity decrease in presence of higher doses of MERIV (10, 20 and 40 µg/g) were 24 ± 5%, 24 ± 5% and 32 ± 0.1%, respectively (n=3, $p<0.05$ each) (Fig. 1A, Table 1).

The analysis of AChE activity was also conducted in biventer cervicis muscle homogenates with different concentrations of MERIV (5, 10 and 20 µg/mL) or neostigmine (0.5µM). In this condition there was also a significative enzyme inhibition, when compared to the control Krebs muscles. The incubation of MERIV (5 µg/mL) decreased the AChE activity in 23 ± 5% (n=3; $p<0.05$). The values of AChE activity for the higher doses of MERIV were 56 ± 5% and 52 ± 6%, respectively (n=3, $p<0.05$ each) (Fig. 1B, Table 1).

3.1.3. Cell viability induced by MERIV on mouse hippocampal slices

Hippocampal mice brain slices were incubated in the presence of MERIV (5, 10, 20, 40 µg/mL) to test the neural cell viability. No significative effect was observed, except at the

lowest dose. When the concentration of 5 μg/mL MERIV was assayed there was a significative increase in cell viability (36 ± 10%) compared to the control HEPES (n=6, $p<0.05$) (Fig. 3A, Table 1). In this set of experiments the application of the Na^+/K^+ pump inhibitor (HORVAT et al., 2006) digoxin (6.5, 13, 26 and 52 nM) induced an effect on cell viability following the same fashion of MERIV (Fig. 3B, Table 1). Thus, the lowest concentration of digoxin induced a significative increase of cell viability (52 ± 7%) when compared to the control HEPES. Higher concentration of digoxin only showed a tendency to decrease the cell viability although not significantly (Fig. 3B, n=6, $p>0.05$ respectively) Table 1.

3.1.4. Neuromuscular blockade induced by MERIV at chick biventer cervicis preparations

At chick nerve-muscle preparations, the incubation of MERIV (5, 10 and 20 μg/mL) induced a time and concentration-dependent activity (Fig. 4). Thus, when the first and second concentrations were assayed, there was a transient increase of muscle twitch tension (60 ± 15% and 46 ± 6%, $p<0.05$ respectively), prior to an irreversible neuromuscular blockade in 120 min recordings (n=5, $p<0.05$) (Fig. 4). At 20 μg/mL MERIV did not induce increase in the twitch tension and provoked a complete neuromuscular blockage in 120 min recordings ($p<0.05$, n=3). At this set of experiments, the incubation of the muscles with 52 nM digoxin or 0.2 nM ouabain mimicked the venom activity by increasing the amplitude of the twitches by 27 ± 6% and 19 ± 4%, respectively and following by a depression of the muscle contraction of 80 ± 4% and 91 ± 5%, respectively in 120 min recordings ($p<0.05$, n=5) (Fig. 5).

To verify if the anti-acetylcholinesterase activity was involved in the neuromuscular activity of MERIV, *biventer cervicis* preparations (BCP) were previously incubated with d-

Tubocurarine (d-Tc 1.45 µM) (n=6). When 1.45 µM d-Tc alone was incubated at BCP there was 60 ± 8% maximum blockage of muscle twitch tension in 120 min recordings (Fig. 6). However, the incubation of d-Tc 1.45 µM 20 min previous the application of MERIV 10 µg/mL has delayed the kinetic of d-Tc´s neuromuscular blockade in ~ 50 min (n=6, $p<0.05$ compared to d-Tc 1.45 µM alone) (Fig. 6). In preparations where d-Tc 1.45 µM was added 20 min after the treatment with MERIV 10 µg/mL there was a reinforcement of the venom neuromuscular blockade that was complete at 80 min (n=6) (Fig.6).

4. DISCUSSION

Toads are endangered animals very acquainted by human anthropic activities including the global warm phenomena (HADDAD, 2011; KATZENBERGER et al., 2012). Despite the ecological reason involved in the need to understand toad venom biological activities, it is wealth of noting that these poisons might be sources of interesting chemical compounds with potential biotechnological applicability. In this work we have shown that the venom of *R. icterica* toad induced both *in vitro* neuroprotection, in mammalian central nervous system, and neuromuscular blockade in chick preparations. The mechanisms involved in these pharmacological profile were investigated by measuring the influence of the venom on cholinesterase and Na^+/K^+ pump activities. The results were discussed in detail herein.

In our experimental conditions, the assay for AChE activity has indicated that MERIV presents a significative anti-AChE effect. At nervous system AChE terminates acetylcholine activity at cholinergic synapses by splitting this neurotransmitter (BEAR; CONNORS; PARADISO, 2002; NELSON; COX, 2010). The nature and distribution of this enzyme has been studied in many invertebrates and vertebrates showing high degree of homology and 3D structures (GREENBLATT; SILMAN; SUSSMAN, 2000). In chick nerve-muscle preparations, the application of MERIV has delayed the neuromuscular blockage of curare, as

do classic anticholinesterase drugs such as neostigmine (LOYOLA et al., 2006). The partial inhibition of the d-Tc neuromuscular activity by MERIV may suggest that at least the anti-AChE activity is involved in the previous increase of muscle twitch tension (GINSBORG; WARRINER, 1960), that precede the irreversible venom neuromuscular blockade. To date, Rostelato and cols. (ROSTELATO-FERREIRA et al., 2011) have demonstrated a similar neuromuscular activity for the venom of *R. shneideri* in avian preparations. In that case, they also observed no facilitation of the twitch tension with a venom concentration above 20 µg/ml, what they have suggested to be probably because the onset of neuromuscular blockade so fast that would masks any facilitation. In this regard, toad venoms are known to exhibit digitalic-like activity (KWAN; PAIUSCO; KOHL, 1992; SAKATE; LUCAS DE OLIVEIRA, 2000; WANG; SUN; HEINBOCKEL, 2014). Indeed, using rat cardiac ventricles sarcolemma, we have demonstrated that MERIV is inhibiting significantly the electrogenic pump, corroborating the literature. In addition, the assays of digoxin and ouabain, at avian neuromuscular preparations, mimicked the neuromuscular activity induced by MERIV. Ouabain, in common with the removal of K^+ from the bathing solution, is a well-known inhibitor of Na^+,K^+-ATPase (MARTIN; MORAD, 1982; VOLKOV et al., 2000; WANG; GEIBEL; GIEBISCH, 1993). Elmqvist and Feldman (ELMQVIST; FELDMAN, 1965a, 1965b) have shown, in a classical work, that ouabain is able to increase the neurotransmitter release in mouse neuromuscular junctions. The same effect was also observed with other digitalis glycosides (BIRKS, 1963). Thus, it is amenable to speculate that both anti-AChE and the blocking of the electrogenic pump activities are possible explanations for explaining MERIV neuromuscular profile.

In our experimental conditions, the venom of *Rhinella icterica* also induced a significative increase of mouse hippocampal cell viability, similarly to digoxin. The MTT assay is a biochemical measure of cell death of brain slices that shows the metabolic conversion of

yellow MTT dye by active mitochondria to form insoluble purple precipitates in live cells. Cell viability in mammalian central nervous system is mostly influenced by toxic conditions which include level of reactive oxygen species, excitotoxicity due to excess of neurotransmitters, necrosis and apoptosis (DAL BELO et al., 2013; DVELA et al., 2012; FRANCO et al., 2009; TAKADA-TAKATORI et al., 2006). In this regard, drugs like anticholinesterases, are good clinical choices for preventing both neuronal death and cognitive impairment in neurodegenerative diseases (TAKADA-TAKATORI et al., 2006). Therefore, despite the clinical relevance of the venom ability to inhibit the electrogenic pump, this pharmacological activity may also reveal its potential as sources for novel drugs in cancer therapy (DVELA et al., 2012).

5. CONCLUSION

Rhinella icterica toad venom induced both inhibitory activities over AChE and Na^+/K^+ pumps. These activities may be directly involved in the neurotoxicity observed in chick neuromuscular junctions and in the increase of cell viability of mouse hippocampal slices. The comprehension of the venom biology is important not only to understand the ecological interactions of *Rhinella icterica* toad, but also reveal the potential biotechnological applications of the poison components.

ACKNOWLEDGMENTS:

The authors thank Michele Corrêa Stach, Rafael Plá Matielo Lemos, Giulianna Echeverria Macedo and Nathane Rosa Rodrigues for the technical assistance with biochemical protocols. The authors also thank the PRONEM/FAPERGS/CNPq 003/2011, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES 063/2010 Toxinologia and PROPESQ/PROPG UNIPAMPA, for financial support. R. S. Oliveira,

Santos, D.S and Almeida, C.G.M. was granted by CAPES. Leal, A.P. was granted by Academic Program Development from UNIPAMPA.

CONFLICT OF INTERESTS: The authors declare that there is no conflict of interests regarding this work.

REFERENCES

[1] F. Heus, R. a. Otvos, R.L.E.G. Aspers, R. van Elk, J.I. Halff, A.W. Ehlers, et al., Miniaturized Bioaffinity Assessment Coupled to Mass Spectrometry for Guided Purification of Bioactives from Road and Cone Snail, Biology (Basel). 3 (2014) 139–156. doi:10.3390/biology3010139.

[2] R. a. Saporito, M.A. Donnelly, T.F. Spande, H.M. Garraffo, A Review of Chemical Ecology in Poison Frogs, Chemoecology. 22 (2012) 159–168. doi:10.1007/s00049-011-0088-0.

[3] G. a. Cunha-Filho, I.S. Resck, B.C. Cavalcanti, C.Ó. Pessoa, M.O. Moraes, J.R.O. Ferreira, et al., Cytotoxic Profile of Natural and Some Modified Bufadienolides from Toad Rhinella schneideri Parotoid Gland Secretion, Toxicon. 56 (2010) 339–348. doi:10.1016/j.toxicon.2010.03.021.

[4] J.M. Sciani, C.B. Angeli, M.M. Antoniazzi, C. Jared, D.C. Pimenta, Differences and Similarities Among Parotoid Macrogland Secretions in South American Toads: A Preliminary Biochemical Delineation, Sci. World J. 2013 (2013) 1–9. doi:10.1155/2013/937407.

[5] A. Siano, P.I. Gatti, M.S. Imaz, E. Zerbini, C. Arturo, R. Lajmanovich, et al., A Comparative Study of the Biological Activity of Skin and Granular Gland Secretions of Leptodactylus latrans and Hypsiboas pulchellus from Argentina, Rec. Nat. Prod. 8 (2014) 128–135.

[6] J.W. Daly, The Chemistry of Poisons in Amphibian Skin., Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9–13. doi:10.1073/pnas.92.1.9.

[7] S. Rostelato-ferreira, C. a D. Belo, G.B. Leite, S. Hyslop, L. Rodrigues-simioni, Presynaptic Neuromuscular Action of a Methanolic Extract from the Venom of Rhinella schneideri Toad, J. Venom. Anim. Toxins Incl. Trop. Dis. 20 (2014) 1–5. doi:10.1186/1678-9199-20-30.

[8] M. Sakate, P.C. Lucas de Oliveira, Toad Envenoming in Dogs: Effects and Treatment, J. Venom. Anim. Toxins. 6 (2000) 1–7. doi:10.1590/S0104-79302000000100003.

[9] Seiichiro Yoshida and Takeshi Sakai, Effects of Bufalin and Related Cardiotonic Steroids in the Neuromuscular Junction, Japan. J. Pharmacol. 23 (1973) 859–869.

[10] Seiichiro Yoshida and Takeshi Sakai, Mechanism of Bufalin-Induced Blockade of Neuromuscular Transmission in Isolated Rat Diaphragm, Japan. J. Pharmacol. 24 (1974) 97–108.

[11] S.C.D. Manika Das, A. Gomes, Isolation, Purification and Partial Chemical Characterization of a Lethal Factor from Common Indian Toad (Bufo melanostictus, Schneider) Skin Extract, Indian J. Exp. Biol. 39 (2001) 781–785.

[12] S. Rostelato-Ferreira, C. a Dal Belo, M.A. da Cruz-Höfling, S. Hyslop, L. Rodrigues-Simioni, Presynaptic Effect of a Methanolic Extract of Toad (Rhinella schneideri) Poison in Avian Neuromuscular Preparation, J. Venom Res. 2 (2011) 32–36.

[13] R.M. Gowda, R.A. Cohen, I.A. Khan, Toad Venom Poisoning: Resemblance to Digoxin Toxicity and Therapeutic Implications, Heart. 89 (2003) 1–2. doi:10.1136/heart.89.4.e14.

[14] H.-Y. Kuo, C.-W. Hsu, J.-H. Chen, Y.-L. Wu, Y.-S. Shen, Life-Threatening Episode After Ingestion of Toad Eggs: a Case Report With Literature Review, Emerg. Med. J. 24 (2007) 215–216. doi:10.1136/emj.2006.044602.

[15] D. Radford, A. Gillies, J. Hinds, P. Duffy, Naturally Occurring Cardiac Glycosides, Med. J. Aust. 144 (1986) 540–543. doi:10.1016/0378-8741(87)90017-1.

[16] D.C. Rohrer, D.S. Fullerton, E. Kitatsuji, T. Nambara, Y. Eiichi, Bufalin*, Acta Cryst. B 38 (1982) 1865–1868.

[17] T. Kwan, A.D. Paiusco, L. Kohl, Digitalis Toxicity Caused by Toad Venom, Chest. 102 (1992) 949–950.

[18] P. Colombo, A. Kindel, G. Vinciprova, L. Krause, Composição e ameaças à conservação dos anfíbios anuros do Parque Estadual de Itapeva, município de Torres, Rio Grande do Sul, Brasil, Biota Neotrop. 8 (2008). doi:10.1590/S1676-06032008000300020.

[19] I. Stefanon, J.R. Cade, A.A. Fernandes, R.F. Ribeiro Junior, G.P. Targueta, J.G. Mill, et al., Ventricular Performance and Na+-K+ ATPase Activity are Reduced Early and Late After Myocardial Infarction in Rats, Braz J Med Biol Res. 42 (2009) 902–911.

[20] M. Bradford, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Anal. Biochem. 254 (1976) 248–254.

[21] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, Biochem. Pharmacol. 7 (1961) 88–95. doi:10.1016/0006-2952(61)90145-9.

[22] G.D. Stürmer, T.C. de Freitas, M. de A. Heberle, D.R. de Assis, L. Vinadé, A.B. Pereira, et al., Modulation of Dopaminergic Neurotransmission Induced by Sublethal Doses of the Organophosphate Trichlorfon in Cockroaches., Ecotoxicol. Environ. Saf. 109 (2014) 56–62. doi:10.1016/j.ecoenv.2014.08.006.

[23] L. Vinadé, R. Rodnight, The Dephosphorylation of Glial Fibrillary Acidic Protein (GFAP) in the Immature Rat Hippocampus is Catalyzed Mainly by a Type 1 Protein Phosphatase, Brain Res. 732 (1996) 195–200.

[24] C.A. Dal Belo, A.P.D.B. Lucho, L. Vinadé, L. Rocha, H. Seibert França, S. Marangoni, et al., In Vitro Antiophidian Mechanisms of Hypericum brasiliense Choisy Standardized Extract: Quercetin-Dependent Neuroprotection, Biomed Res. Int. 2013 (2013) 1–6. doi:10.1155/2013/943520.

[25] B.L. Ginsborg, J. Warriner, The Isolated Chick Biventer Cervicis Nerve-Muscle Preparation, Brit. J. Pharmacol. 15 (1960) 410–411.

[26] A. Horvat, T. Momic, A. Banjac, S. Petrovic, G. Nikezic, M. Demajo, Selective Inhibition of Brain Na , K-ATPase by Drugs, Physiol. Res. 55 (2006) 325–338.

[27] D. Conrado, D.E.A. Munhoz, M.C. dos Santos, R.F.L. MEllo, V.B. Silva, Vulnerabilidades às Mudanças Climáticas, IIEB. (2000) 1–10.

[28] M. Katzenberger, M. Tejedo, H. Duarte, F. Marangoni, J.F. Beltrán, Especial Mudanças Ambientais, Rev. Da Biol. 8 (2012) 25–32. www.ib.usp.br/revista (accessed February 4, 2016).

[29] C.F.B. Haddad, ANFÍBIOS - Uma Análise da Lista Brasileira de Anfíbios Ameaçados de Extinção, ICMBIO. 2 (2011) 287–288. http://www.icmbio.gov.br/portal/images/stories/biodiversidade/fauna-brasileira/livro-vermelho/volumeII/Anfibios.pdf (accessed January 24, 2016).

[30] D.L. Nelson, M.M. Cox, Principles of Biochemistry, 5ª ed., Sara Tenney, New York, 2010. doi:10.1016/0020-711X(94)90020-5.

[31] M.F. Bear, W.B. Connors, M.A. Paradiso, Neurociências: Desvendando o Sistema Nervoso, Artmed Editora S.A., Porto Alegre, 2002.

[32] H.M. Greenblatt, I. Silman, J.L. Sussman, Structural Studies on Vertebrate and Invertebrate Acetylcholinesterases and Their Complexes With Functional Ligands, Drug Dev. Res. 50 (2000) 573–583.

[33] Y.C.S. Loyola, A. de F. de A. Braga, G.M.B. Potério, S.R. de Sousa, S.C.A.F. Fernandes, F.S. da S. Braga, Influência da Lidocaína no Bloqueio Neuromuscular Produzido pelo Rocurônio. Estudo em Preparação Nervo Frênico-Diafragma de Rato, Rev Bras Anestesiol. 56 (2006) 147–156.

[34] Z.-J. Wang, L. Sun, T. Heinbockel, Resibufogenin and Cinobufagin Activate Central Neurons through an Ouabain-Like Action, PLoS One. 9 (2014) e113272. doi:10.1371/journal.pone.0113272.

[35] G. Martin, M. Morad, Activity-Induced Potassium Accumulation and its Uptake in Frog Ventricular Muscle, J. Physiol. 328 (1982) 205–227.

[36] W.H. Wang, J. Geibel, G. Giebisch, Mechanism of Apical K+ Channel Modulation in Principal Renal Tubule Cells. Effect of Inhibition of Basolateral Na+,K+-ATPase, J. Gen. Physiol. 101 (1993) 673–694.

[37] E.M. Volkov, L.F. Nurullin, I. Svandová, E.E. Nikolscky, F. VysKocil, Participation of Electrogenic Na+,K+,ATPase in the Membrane Potential of Earthworm Body Wall Muscles, Physiol. Res. 49 (2000) 481–484.

[38] T. Clausen, Na+-K+ Pump Regulation and Skeletal Muscle Contractility., Physiol. Rev. 83 (2003) 1269–1324. doi:10.1152/physrev.00011.2003.

[39] B.Y.D. Elmqvist, A.D.D.S. Feldman, Calcium Dependence of Spontaneous Acetylcholine Release at Mammalian Motor Nerve Terminals, J. Physiol. 181 (1965) 487–497.

[40] B.Y.D. Elmqvist, D.S. Feldman, Effects of Sodium Pump Inhibitors on Spontaneous Acetylcholine Release at the Neuromuscular Junction, J. Physiol. 181 (1965) 498–505.

[41] R.I. Birks, The Role of Sodium Ions in the Metabolism of Acetylcholine, Can. J. Biochem. Physiol. 41 (1963) 2573–2597.

[42] M. Dvela, H. Rosen, H.C. Ben-Ami, D. Lichtstein, Endogenous Ouabain Regulates

Cell Viability, Am J Physiol Cell Physiol. 302 (2012) C442–C452. doi:10.1152/ajpcell.00336.2011.

[43] J.L. Franco, T. Posser, P.R. Dunkley, P.W. Dickson, J.J. Mattos, R. Martins, et al., Methylmercury Neurotoxicity is Associated With Inhibition of the Antioxidant Enzyme Glutathione Peroxidase, Free Radic. Biol. Med. 47 (2009) 449–457. doi:10.1016/j.freeradbiomed.2009.05.013.

[44] Y. Takada-Takatori, T. Kume, M. Sugimoto, H. Katsuki, H. Sugimoto, A. Akaike, Acetylcholinesterase Inhibitors Used in Treatment of Alzheimer's Disease Prevent Glutamate Neurotoxicity Via Nicotinic Acetylcholine Receptors and Phosphatidylinositol 3-kinase Cascade, Neuropharmacology. 51 (2006) 474–486. doi:10.1016/j.neuropharm.2006.04.007.

Figure Legends

Figure 1: Inhibition of acetylcholinesterase (AChE) activity induced by *Rhinella icterica* toad venom (MERIV). Panel A: Representative graph of acetylcholinesterase inhibition in cockroaches' brain homogenates after 6 hours exposure to MERIV (5, 10, 20 and 40 µg/g) and neostigmine (0.5 µM). Panel B: acetylcholinesterase activity in chick *biventer cervicis* muscle after MERIV (5, 10 and 20µg/mL) treatments and neostigmine (0.5 µM), with indirect stimulation, in comparison with those obtained only with Krebs control. Data were expressed as mean ± S.E.M. Significance at **p*<0.05 in comparison to Saline (control 100%).

Figure 2: Inhibition of rat cardiac Na^+,K^+-ATPase activity induced by *Rhinella icterica* toad venom (MERIV). Na^+,K^+-ATPase activity was assayed by measuring Pi liberation by reacting initiated by adding ATP. The specific activity was reported as nmol Pi released per min per mg of protein. Results represent Mean ± S.E.M. (n=4). Significance *p<0.05 compared to control.

Figure 3: Effects of *Rhinella icterica* toad venom (MERIV) and digoxin on the cell viability of hippocampal slices. Panel A: Hippocampal slices were incubated with MERIV (5, 10, 20 and 40 µg/mL) during 60 min. Note that the concentration of 5 µg/mL induced a significant increase in the cell viability (n=6, p<0.05). Panel B: Hippocampal slices were incubated with digoxin (6.5, 13, 26 and 52 nM) during 60 min. Note that the concentration of 6.5 and 13 nM induced a significative increase in cell viability (n=6, p<0.05). Data were expressed as mean ± S.E.M. Significance at **p*<0.05 compared to Hepes (control 100%).

Figure 4: Concentration-response of MERIV at chick muscle-nerve preparations. BCP muscle-nerve preparation was indirectly stimulated (0.1 Hz, 0.2ms, 3-10V) during 120 min. Panel A: In the graphic each point represents the mean ± S.E.M. (n = 3-5). Panel B: Representative traces of the activity of MERIV 10 µg/mL on evoked responses of BCP muscle-nerve preparation. Note that the venom (5 and 10 µg/mL) induced a transitory increase of muscle twitch-tension followed by an irreversible neuromuscular blockade after 30min recordings. Significance at **p*<0.05, compared to Krebs control.

Figure 5: Comparison of the *Rhinella icterica* toad venom (MERIV) neuromuscular activity with different pharmacological treatments in *biventer cervicis* muscle nerve preparations (BCP). Panel A: Shows the activity induced by 10 µg/mL MERIV, 52 nM digoxin and 0.2 nM ouabain at BCP muscle-nerve preparations in 120min (0.1Hz, 0.2ms, 5-8V). In the graphic each point represents the mean ± S.E.M. (n = 4-6). Note that ouabain and digoxin have a similar activity compared to the MERIV. Panel B: representative traces of the effect of 0.2 nM ouabain on evoked responses at BCP preparations. Significance at **p*<0.05 compared to MERIV.

Figure 6: Protective activity of *Rhinella icterica* toad venom (MERIV) against the neuromuscular blockade induced by d-Tubocurarine (d-Tc) at chick muscle-nerve preparations (BCP). BCP was indirectly stimulated (0.1 Hz, 0.2ms, 3-10V) during 120 min. Panel A: Shows the activity induced by 1.45 µM d-Tc in BCP. Panel B: Demonstrates the muscle response of d-Tc preincubated and post incubated with MERIV 10 µg/mL on evoked responses at BCP. Note that the addition of d-Tc prior to MERIV partially prevented the neuromuscular blockade induced by the curare. In the graphic each point represents the Mean ± S.E.M. (n = 3-5). Significance at **p*<0.05, compared to d-Tc.

Table 1: Biochemical analysis of *Rhinella icterica* toad venom (MERIV) over acetylcholinesterase activity of cockroach brain homogenates, chick *biventer cervicis* muscles and to the hippocampal cell viability. The data with the venom was compared to the isolated treatment with digoxin and neostigmine. Note that the venom increased the cell viability as do digoxin.* indicate significance at $p<0.05$.

Figure 1:

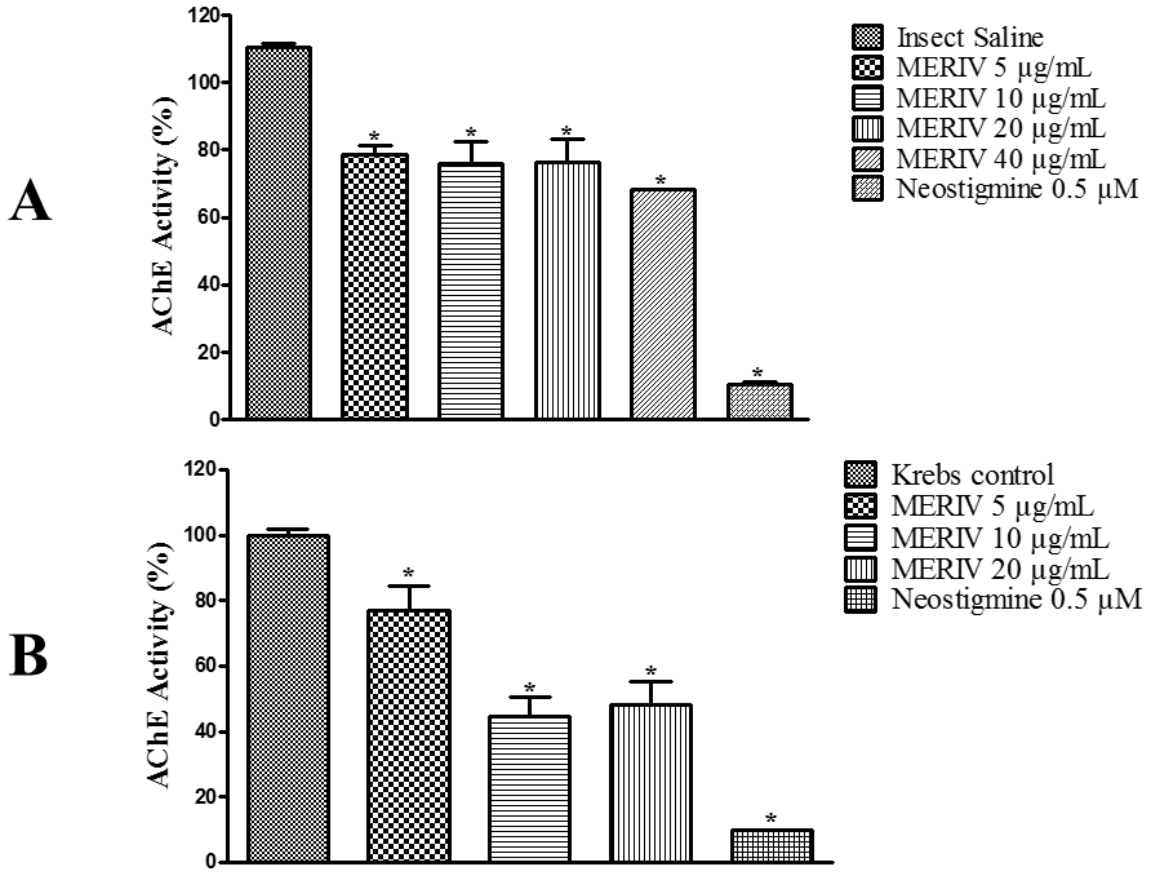

A
AChE Activity (%)
120
100
80
60
40
20
0
Insect Saline
MERIV 5 µg/mL
MERIV 10 µg/mL
MERIV 20 µg/mL
MERIV 40 µg/mL
Neostigmine 0.5 µM
B
AChE Activity (%)
120
100
80
60
40
20
0
Krebs control
MERIV 5 µg/mL
MERIV 10 µg/mL
MERIV 20 µg/mL
Neostigmine 0.5 µM

Figure 2:

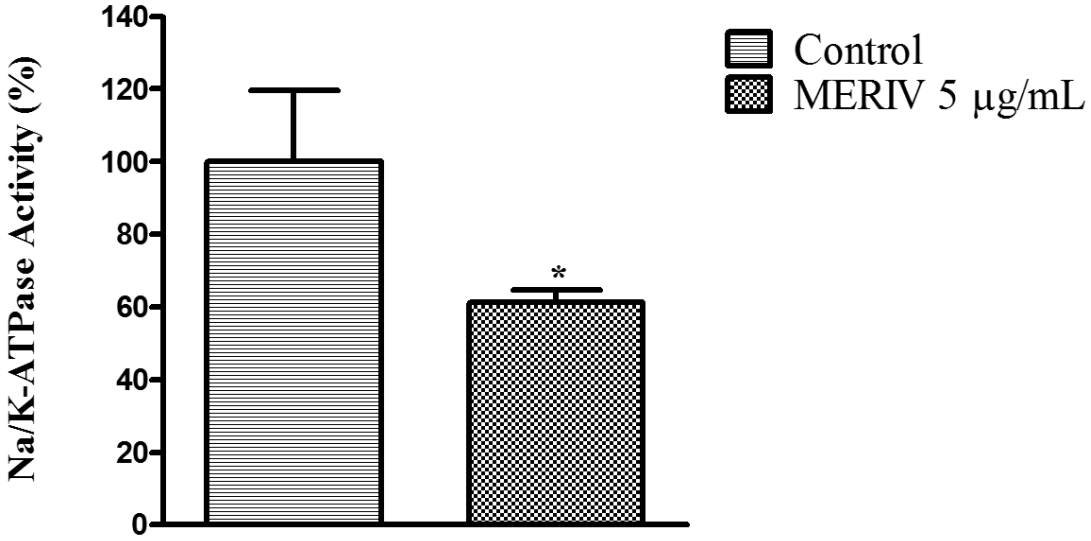

Na/K-ATPase Activity (%)
140
120
100
80
60
40
20
0
*
Control
MERIV 5 µg/mL

Figure 3:

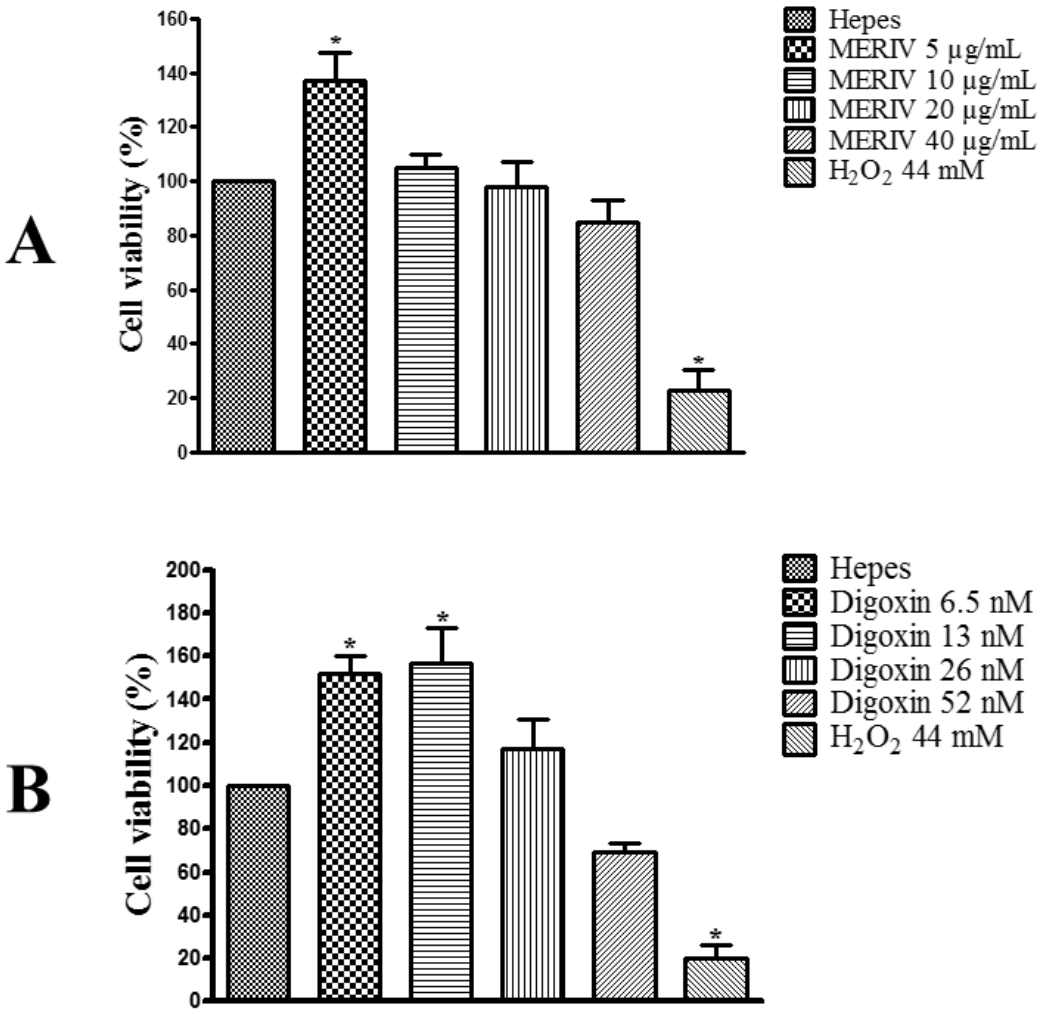

A
Cell viability (%)
160
140
120
100
80
60
40
20
0
*
*
Hepes
MERIV 5 µg/mL
MERIV 10 µg/mL
MERIV 20 µg/mL
MERIV 40 µg/mL
H2O2 44 mM
B
Cell viability (%)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
Hepes
Digoxin 6.5 nM
Digoxin 13 nM
Digoxin 26 nM
Digoxin 52 nM
H2O2 44 mM

Figure 4:

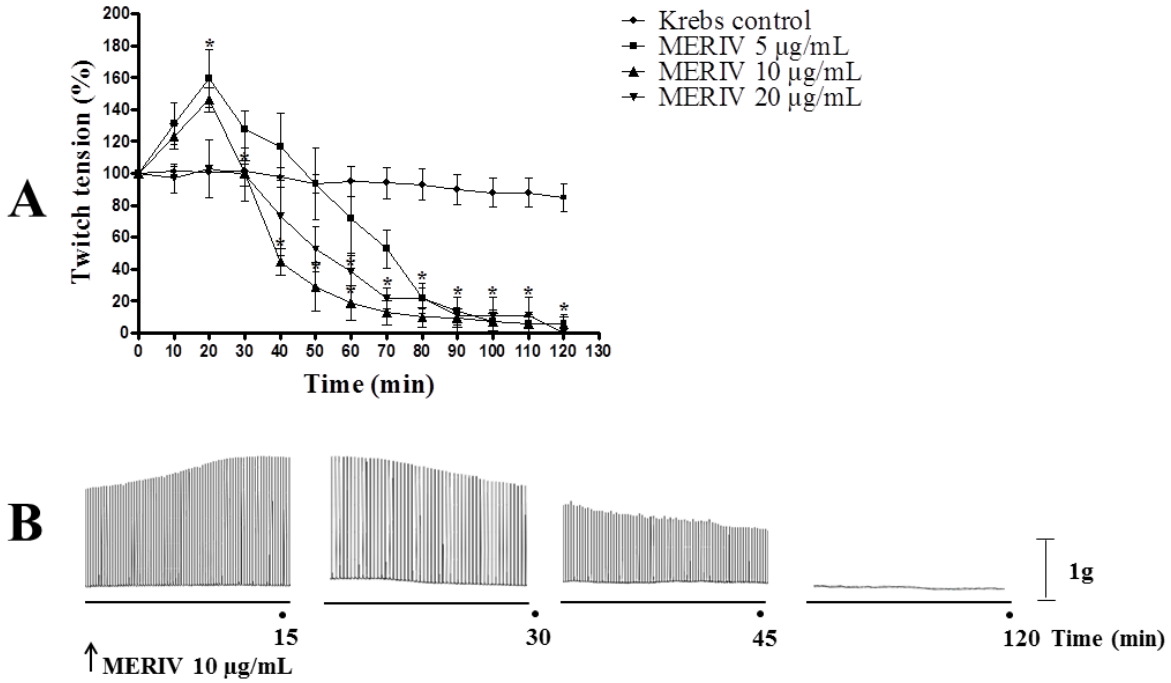

A
Twitch tension (%)
Time (min)
Krebs control
MERIV 5 µg/mL
MERIV 10 µg/mL
MERIV 20 µg/mL
B
1g
15
30
45
120 Time (min)
MERIV 10 µg/mL

Figure 5:

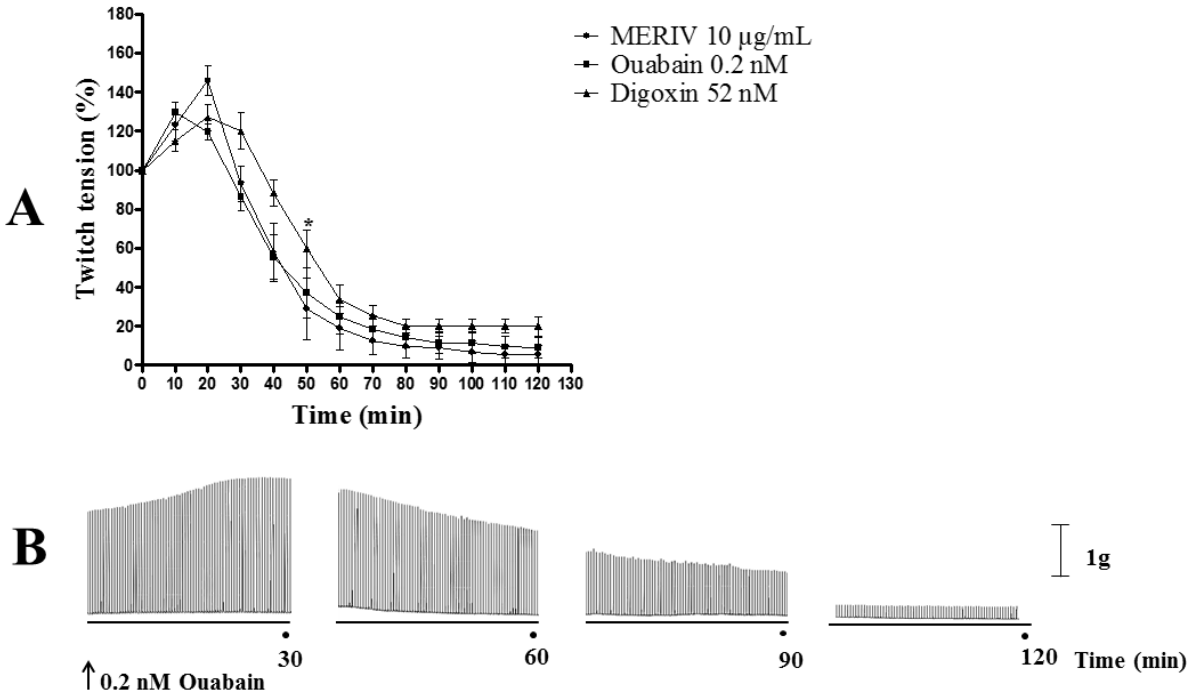

A
Twitch tension (%)
Time (min)
MERIV 10 µg/mL
Ouabain 0.2 nM
Digoxin 52 nM
B
1g
0.2 nM Ouabain
30
60
90
120
Time (min)

Figure 6:

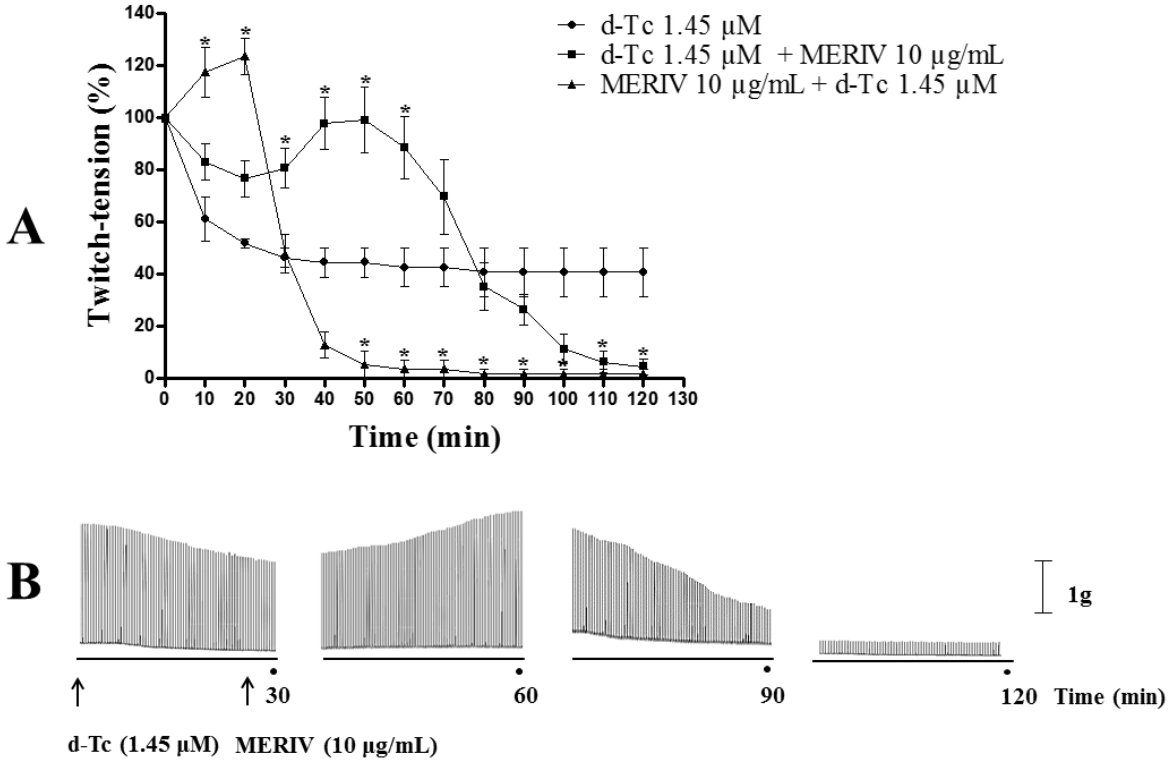

A
Twitch-tension (%)
Time (min)
d-Tc 1.45 µM
d-Tc 1.45 µM + MERIV 10 µg/mL
MERIV 10 µg/mL + d-Tc 1.45 µM
B
1g
30
60
90
120
Time (min)
d-Tc (1.45 µM)
MERIV (10 µg/mL)

Table 1:

	AChE activity in cockroaches (mU/mg protein)	Inhibition (%)	AChE activity in chick' muscles (mU/mg protein)	Inhibition (%)	Cell viability (%)
Control	221.96	0	48.13	0	100
MERIV 5 µg/mL	174.52 *	21.37 *	37.08 *	22.95 *	136.91 *
MERIV 10 µg/mL	168.31 *	24.17 *	21.38 *	55.57 *	104.75
MERIV 20 µg/mL	183.67 *	23.62 *	23.20 *	51.80 *	97.91
MERIV 40 µg/mL	160.62 *	31.78 *	-	-	84.76
Digoxin 6.5 nM	-	-	-	-	152.25 *
Digoxin 13 nM	-	-	-	-	156.91 *
Digoxin 26 nM	-	-	-	-	116.92
Digoxin 52 nM	-	-	-	-	69.58
Neostigmine 0.5 µM	22.88 *	89.69 *	4.66 *	90.32 *	-

5. CONSIDERAÇÕES FINAIS

Com base nos dados obtidos no presente estudo, podemos considerar:

- Nas menores concentrações, MERIV (5 μg/mL) e Digoxina (6,5 e 13 nM) demonstraram aumento da viabilidade celular em fatias de hipocampo de camundongos. Condizente com os resultados encontrados na literatura com glicosídeos cardiotônicos.

- MERIV inibiu significativamente a atividade da acetilcolinesterase em todas as doses avaliadas, tanto em invertebrados (cérebro de baratas), quanto em músculos utilizados na preparação *biventer cervicis* de pintainho.

- Em preparação *biventer cervicis* de pintainho, MERIV 5 e 10 μg/mL induziram bloqueio neuromuscular precedido de facilitação, possivelmente pela atividade anticolinesterasica e por inibição da Na^+,K^+-ATPase.

- Em preparação *biventer cervicis* de pintainho, a atividade de MERIV (10 μg/mL) foi similar a dos inibidores da Na^+,K^+-ATPase, digoxina (52 nM) e Ouabaína (0,2 nM), sugerindo que MERIV inibe a Na^+,K^+-ATPase.

- MERIV prorrogou o bloqueio neuromuscular induzido por d-Tubocurarina, demonstrando atividade similar a de anticolinesterásicos.

- MERIV inibiu significativamente a atividade da Na^+,K^+-ATPase em tecido cardíaco de ratos, comprovando que estão presentes no veneno toxinas que atuam na Na^+,K^+-ATPase.

- Este trabalho contribui para caracterizar a atividade biológica do veneno de sapos da espécie *R. icterica* em sistema nervoso central e periférico de vertebrados. As atividades anticolinesterasica e digitalic-like presentes no veneno demonstram a importância das toxinas presentes no veneno desta espécie e sua aplicabilidade terapêutica.

6. PERSPECTIVAS

Com base nos resultados obtidos neste estudo, pretendemos:

- Contribuir para o conhecimento do mecanismo da neurotoxicidade por meio da identificação bioquímica e farmacológica dos compostos ativos isolados do veneno de *R. icterica* sobre o sistema nervoso de vertebrados.

- Isolar e purificar as toxinas presentes neste veneno com atividade anticolinesterásica e/ou que apresentem ação sobre a Na^+,K^+-ATPase.

- Avaliar o mecanismo de ação dos compostos isolados do veneno junto à junção neuromuscular de vertebrados, e investigar a sua farmacologia com ênfase na identificação dos possíveis sítios ativos.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ABDEL-RAHMAN, M. A.; AHMED, S. H.; NABIL, Z. I. In Vitro Cardiotoxicity and Mechanism of Action of the Egyptian Green Toad Bufo viridis Skin Secretions. **Toxicology in vitro**, v. 24, n. 2, p. 480–485, 2010.

ARISI, G. M.; NEDER, L.; MOREIRA, J. E. O Ciclo Da Vesícula Sináptica : Panorama Molecular*. **Medicina, Ribeirão Preto**, v. 34, p. 154–169, 2001.

BAGROV, A. Y.; SHAPIRO, J. I.; FEDOROVA, O. V. Endogenous Cardiotonic Steroids: Physiology, Pharmacology, and Novel Therapeutic Targets. **Pharmacological reviews**, v. 61, n. 1, p. 9–38, 2009.

BARRAVIERA, B. **Venenos Animais: Uma Visão Integrada**. Rio de Janeiro: Editora de Publicações Científicas, 1994.

BEAR, M. F.; CONNORS, W. B.; PARADISO, M. A. **Neurociências: Desvendando o Sistema Nervoso**. Porto Alegre: Artmed Editora S.A., 2002. v. 2.

BUAINAIN, R. P.; MOURA, L. S.; OLIVEIRA, A. S. B. Fasciculação. **Rev. Neurociências**, v. 8, n. 1, p. 31–34, 2000.

CHEN, J. Q. et al. Sodium/Potasium ATPase (Na+, K+-ATPase) and Ouabain/Related Cardiac Glycosides: A New Paradigm for Development of Anti- Breast Cancer Drugs? **Breast Cancer Research and Treatment**, v. 96, n. 1, p. 1–15, 2006.

CHEN, K. K.; KOVARIOVÁ, A. Pharmacology and Toxicology of Toad Venom. **Journal of Pharmaceutical Sciences**, v. 56, n. 12, p. 1535–1541, 1967.

CHOI, Y. H.; HONG, S.-H. Bufalin Induces Apoptosis Through Activation of Both the Intrinsic and Extrinsic athways in Human Bladder Cancer Cells. **Oncology Reports**, v. 27, n. 15, p. 114–120, 2012.

CUNHA-FILHO, G. A. et al. Cytotoxic Profile of Natural and Some Modified Bufadienolides from Toad Rhinella schneideri Parotoid Gland Secretion. **Toxicon**, v. 56, n. 3, p. 339–348, 2010.

DALY, J. W. The Chemistry of Poisons in Amphibian Skin. **Proceedings of the National Academy of Sciences of the United States of America**, v. 92, n. 1, p. 9–13, 1995.

DALY, J. W.; SPANDE, T. F.; GARRAFFO, H. M. Alkaloids from Amphibian Skin: A Tabulation of Over Eight-Hundred Compounds. **Journal of Natural Products**, v. 68, n. 10, p. 1556–1575, 2005.

DANIEL, L. et al. Regulation of the Sperm EGF Receptor by Ouabain Leads to Initiation of the Acrosome Reaction. **Developmental Biology**, v. 344, n. 2, p. 650–657, 2010.

DE ROBERTIS, E. M. F.; HIB, J. **Bases da Biologia Celular e Molecular**. 4a. ed. Rio de Janeiro: GUANABARA KOOGAN, 2006.

DENG, L.-J. et al. Hellebrigenin Induces Cell Cycle Arrest and Apoptosis in Human Hepatocellular Carcinoma HepG2 Cells Through Inhibition of Akt. **Chemico-biological interactions**, v. 219, p. 184–194, 2014. 41.

DVELA, M. et al. Endogenous Ouabain Regulates Cell Viability. Am J Physiol Cell Physiol, v. 302, p. C442–C452, 2012.

FROST, D. R. 2015. Amphibian Species of the World: an Online Reference. Version 6.0 (20/01/2016). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

GOWDA, R. M.; COHEN, R. A.; KHAN, I. A. Toad Venom Poisoning: Resemblance to Digoxin Toxicity and Therapeutic Implications. Heart (British Cardiac Society), v. 89, n. 4, p. 1–2, 2003.

HORVAT, A. et al. Selective Inhibition of Brain Na , K-ATPase by Drugs. Physiol. Res., v. 55, p. 325–338, 2006.

HUGHES, B. W.; KUSNER, L. L.; KAMINSKI, H. J. Molecular Architecture of the Neuromuscular Junction. Muscle & nerve, v. 33, n. 4, p. 445–461, 2006.

JORGENSEN, P. L.; HÅKANSSON, K. O.; KARLISH, S. J. D. Structure and Mechanism of Na,K-ATPase: Functional Sites and Their Interactions. Annual Review of Physiology, v. 65, n. 1, p. 817–849, 2003.

KAMBOJ, A.; RATHOUR, A.; KAUR, M. Bufadienolides and Their Medicinal Utility : a Review. International Journal of Pharmacy and Pharmaceutical Sciences, v. 5, n. 4, p. 20–27, 2013.

KATZ, R. L. Neuromuscular Effects of Diethyl Ether and Its Interaction with Succinylcholine and d-Tubocurarine. Anesthesiology, v. 27, n. 1, p. 52–63, 1966.

KOTOVA, O. et al. Metabolic and Signaling Events Mediated by Cardiotonic Steroid Ouabain in Rat Skeletal Muscle. Cell and Molecular Biology, v. 52, n. 8, p. 48–57, 2006.

KUO, H.-Y. et al. Life-Threatening Episode After Ingestion of Toad Eggs: a Case Report With Literature Review. Emerg. Med. J., v. 24, n. 3, p. 215–216, 2007.

KWAN, T.; PAIUSCO, A. D.; KOHL, L. Digitalis Toxicity Caused by Toad Venom. Chest, v. 102, n. 3, p. 949–950, 1992.

LEMA, THALES DE; MARTINS, L. A. Anfíbios do Rio Grande do Sul: Catálogo, Diagnoses, Distribuição, Iconografia. Porto Alegre: EDIPUCRS, 2011.

LENT, R. CEM BILHÕES DE NEURÔNIOS? CONCEITOS FUNDAMENTAIS DE NEUROCIÊNCIA. 2a. ed. Rio de Janeiro: ATHENEU, 2010.

LI, Z.; XIE, Z. The Na/K-ATPase/Src Complex and Cardiotonic Steroid-Activated Protein Kinase Cascades. Pflügers Archiv - European Journal of Physiology, v. 457, n. 3, p. 635–644, 2009.

MANIKA DAS, S. C. D.; GOMES, A. Isolation, Purification and Partial Chemical Characterization of a Lethal Factor from Common Indian Toad (Bufo melanostictus, Schneider) Skin Extract. Indian Journal of Experimental Biology, v. 39, n. 8, p. 781–785, 2001. 42.

MELLA, E. A. C.; AMBIEL, C. R.; PRADO, W. A. DO. A Potenciação Pós-Tetânica e a Transmissao Neuromuscular. Arq. Ciênc. Saúde Unipar, v. 4, n. 3, p. 277–281, 2000.

MORENO Y BANULS, L. et al. Hellebrin and Its Aglycone Form Hellebrigenin Display Similar In Vitro Growth Inhibitory Effects in Cancer Cells and Binding Profiles to the Alpha Subunits of the Na+,K+-ATPase. Molecular cancer, v. 12, n. 33, p. 1–14, 2013.

PALTY, R.; SEKLER, I. The Mitochondrial Na+/Ca2+ Exchanger. Cell Calcium, v. 52, n. 1, p. 9–15, 2012.

RADFORD, D. et al. Naturally Occurring Cardiac Glycosides. The Medical Journal of Australia, v. 144, n. 3, p. 540–543, 1986.

RANG, H. P. et al. FARMACOLOGIA. 5a. ed. Rio de Janeiro: Elsevier, 2005.

ROHRER, D. C. et al. Bufalin*. Acta Cryst., v. B 38, p. 1865–1868, 1982.

ROMAN, I. et al. Síntese de Dihidropiridinas Via Reação Multicomponente de Hantzsch, Aplicando os Conceitos da Química Verde. Perspectiva, v. 36, n. 135, p. 93–99, 2012.

ROSTELATO-FERREIRA, S. et al. Presynaptic Effect of a Methanolic Extract of Toad (Rhinella schneideri) Poison in Avian Neuromuscular Preparation. J. Venom Res., v. 2, p. 32–36, 2011.

ROSTELATO-FERREIRA, S. et al. Presynaptic Neuromuscular Action of a Methanolic Extract from the Venom of Rhinella schneideri Toad. Journal of Venomous Animals and Toxins including Tropical Diseases, v. 20, n. 30, p. 1–5, 2014.

SAKATE, M.; LUCAS DE OLIVEIRA, P. C. Toad Envenoming in Dogs: Effects and Treatment. Journal of Venomous Animals & Toxins, v. 6, n. 1, p. 1–7, 2000.

SAPORITO, R. A et al. Formicine Ants: An Arthropod Source for the Pumiliotoxin Alkaloids of Dendrobatid Poison Frogs. PNAS, v. 101, n. 21, p. 8045–8050, 2004.

SAPORITO, R. A. et al. A Review of Chemical Ecology in Poison Frogs. Chemoecology, v. 22, n. 3, p. 159–168, 2012.

SCIANI, J. M. et al. Differences and Similarities Among Parotoid Macrogland Secretions in South American Toads: A Preliminary Biochemical Delineation. The Scientific World Journal, v. 2013, p. 1–9, 2013.

SEGALLA, M. V et al. Brazilian Amphibians : List of Species. Herpetologia Brasileira, v. 3, n. 2, p. 37–48, 2014.

SEIICHIRO YOSHIDA AND TAKESHI SAKAI. Effects of Bufalin and Related Cardiotonic Steroids in the Neuromuscular Junction. Japan. J. Pharmacol., v. 23, p. 859–869, 1973.

SEIICHIRO YOSHIDA AND TAKESHI SAKAI. Mechanism of Bufalin-Induced Blockade of Neuromuscular Transmission in Isolated Rat Diaphragm. Japan. J. Pharmacol., v. 24, p. 97–108, 1974.

SHAMAGIAN, L. G. et al. Evolución a Largo Plazo de la Prescripción de Fármacos en Pacientes Hospitalizados por Insuficiencia Cardíaca Congestiva. Influencia del Patrón de Disfunción. Rev Esp Cardiol., v. 58, n. 4, p. 381–388, 2005. 43.

SIANO, A. et al. A Comparative Study of the Biological Activity of Skin and Granular Gland Secretions of Leptodactylus latrans and Hypsiboas pulchellus from Argentina. Rec. Nat. Prod., v. 8, n. 2, p. 128–135, 2014.

SILVERTHORN, D. U. Fisiologia Humana - Uma Abordagem Integrada. 5a. ed. Porto Alegre: Artmed, 2010.

SONNE, L. et al. Intoxicação por Veneno de Sapo em um Canino. Ciência Rural, v. 38, n. 6, p. 1787–1789, 2008.

SOUSA, S. R. et al. Influência da Nifedipina no Bloqueio Neuromuscular Produzido pelo Atracúrio e pelo Cisatracúrio. Estudo em Preparações Nervo Frênico-Diafragma de Rato. Rev Bras de Anestesiol, v. 56, n. 2, p. 157–167, 2006.

TAKAI, N. et al. Bufalin, a Traditional Oriental Medicine, Induces Apoptosis in Human Cancer Cells. Asian Pacific Journal of Cancer Prevention, 2012.

TEMPONE, A. G. et al. Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad Rhinella jimi parotoid macrogland secretion. Toxicon, v. 52, n. 1, p. 13–21, 2008.

YANG, Q. et al. Angel of Human Health : Current Research Updates in Toad Medicine. Am J Transl Res, v. 7, n. 1, p. 1–14, 2015.

YIN, P. H. et al. Anti-Tumor Activity and Apoptosis-Regulation Mechanisms of Bufalin in Various Cancers: New Hope for Cancer Patients. Asian Pacific Journal of Cancer Prevention, v. 13, n. 11, p. 5339–5343, 2012.

8. ANEXOS

8.1. Aprovação do CEUA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

PROTOCOLO Nº 037/2012

Título:
MECANISMOS ENVOLVIDOS NA NEUROPROTEÇÃO: ÊNFASE NOS SISTEMAS GLUTAMATÉRGICO, PURINÉRGICO, COLINÉRGICO E DOPAMINÉRGICO

Pesquisador: Lucia Helena do Canto Vinadé

Campus: São Gabriel

Telefone: (55) 3232 6075 R: 2256

E-mail: luciavinade@unipampa.edu.br

Após a análise detalhada do projeto de pesquisa a relatoria da CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa

8.2. Participação em Congresso Internacional

We hereby certify that the abstract entitled

NEUROBIOLOGY OF RHINELLA ICTERICA TOAD VENOM IN MICE AND CHIKENS

by authors: RAQUEL SOARES OLIVEIRA (UNIPAMPA), ALLAN PINTO LEAL (UNIPAMPA), CARLOS GABRIEL MOREIRA DE ALMEIDA (UNIPAMPA), TIAGO GOMES DOS SANTOS (UNIPAMPA), CHÁRISTON ANDRÉ DAL BELO (UNIPAMPA,UFSM), LÚCIA HELENA DO CANTO VINADÉ (UNIPAMPA)

was presented during the 23rd International Congress of the IUBMB and 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq) in Foz do Iguaçu, Paraná, Brazil, on August 24th to 28th, 2015.

Glaucius Oliva
Chair of the 23rd IUBMB Congress

Jerson Lima Silva
President of SBBq

8.3. Artigo submetido para o periódico Journal of Neuroscience Research (ISSN: 1097-4547)

Electrophysiological and Biochemical Investigation of the Stimulating Activity of *Canavalia ensiformis* Urease in Mammalian Nervous System

Carlos Gabriel Moreira de Almeida[1,2], Raquel Soares Oliveira[1], Carolina Nunes da Silva[4], Rafael Stuani Floriano[3], Maria Elena de Lima[4], João Pereira Leite[6], Edward G. Rowan[5], Alexandre Pinto Corrado[6], Lucia Vinade[1], Celia Regina Carlini[2,8], Chariston André Dal Belo[1,2,7,8*]

[1]LANETOX, Universidade Federal do Pampa, UNIPAMPA, São Gabriel, Brazil; [2]LaNeurotox,BrainInstitute, Pontificia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil; [3]Faculdade de Ciências Médicas, Departamento de Farmacologia,Universidade Estadual de Campinas, UNICAMP, Campinas, Brazil; [4]Laboratório de Venenos e Toxinas Animais (LVTA), Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brazil; [5]SIPBS, University of Strathclyde, Glasgow, UK; [6]FMRP, University of São Paulo, Ribeirão Preto, SP, Brazil; [7]PPGBTox, Universidade Federal de Santa Maria, UFSM, Santa Maria, Brazil; [8]Center of Biotechnology, Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre Brazi[l.].

*Chariston André Dal Belo, PhD. Address Federal University of Pampa (UNIPAMPA) Av. Antônio Trilha, 1847 – Centro, CEP 97300-000, São Gabriel-RS, Brazil. Phone/Fax: +55 55 3232-6075;
e-mail:charistonbelo@unipampa.edu.br

Abstract

The *Canavalia ensiformis* urease (Jack Bean Urease) has a 90,770 kDa polypeptide containing 840 aminoacid residues. JBU is known to exhibit insecticidal and fungicidal activities. When administered endovenously in mammalians, it induces tonic clonic convulsions culminating in the death of the animals. This mechanisms involved in the excitatory activity of JBU has not been elucidated so far. In this work, we sought to investigate the central and peripheral electrophysiological patterns of Jack Bean Urease in rodents, *in vivo* and *in vitro*, as well as the biochemical correlation of cell viability and glutamate release. In the biochemical assays, JBU induced increase in L-Glutamate release in

rat cortical sinaptossomes, with no alteration of mice hippocampal cell viability. The electrophysiological assays, showed that JBU induce a significant decrease on mice sciatic nerve compound action potentials (CAP), and spike-wave discharges (SWD) similar to “petit mal” seizures when injected directly in the hippocampus (10 nM). The decrease in CAP amplitude is related to a blockage of voltage-gated sodium channels, since it was not affected by the concomitant application of tetrodotoxin. Our results show that JBU exerts an effect of spike wave discharges-like activity over the mammalian central nervous system. This later result suggests an involvement of T-type voltage gated calcium channels in the excitatory activity of JBU. The blockade of mouse sciatic nerve compound action potential conduction corroborates the excitatory activity of the urease upon the mammalian central nervous system.

Keywords: Jack Bean Urease, blockade of voltage-gated sodium channels, L-Glu release, absence crises.

1. Introduction

Ureases (EC 3.5.1.5 aminohydrolases) are nickel-dependent metalloenzymes that hydrolyze the interconvertion of urea into two molecules of ammonia and one of carbon dioxide (DIXON et al., 1975). Ureases are synthesized by plants, bacteria and fungi, but not by animals (Mobley et al. 1995; Krajewska 2009). It is important to note, that although composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi exhibit high homology of amino acid sequences (KRAJEWSKA, 2009).

The Canavalia ensiformis urease (Jack Bean Urease, JBU) has an 840-amino acid residue polypeptide chain, and a molecular mass of 90,770 kDa. JBU is known to exhibit

insecticidal (Ferreira-DaSilva et al. 2000; Martinelli et al. 2014) and fungicidal (Becker-Ritt et al. 2007; Carlini and Polacco 2008) activities.

In mammalians, the toxic effect of *Canavalia ensiformis* crude extract has been assessed in rodents and showed to induce dyspnea, ataxia, hypothermia, coma, tonic convulsions and death (Carlini et al., 1984). Its toxicity to murines is mainly devoid to the presence of a toxic peptide named Canatoxin (CNTX) (Carlini et. al., 1984). The lethal and convulsive effect of CNTX occurs in mice and rats and is observed after endogenous administration (Carlini et al., 1984). The same effects are observed with JBU, although they appear only by endovenous injection (FOLLMER et al., 2004). The observation of Straub phenomena and the ability of reserpine, a drug which inhibits the vesicular monoamines transporter (VMAT), to decrease the threshold of seizures, suggested the central nervous system as the target for CNTX convulsive activity (Carlini et al., 1984). On the other hand, it was also observed that the disruption of spinal column at T5 region, was able to inhibit the seizures related to the hind limbs (lower limbs). In this regard, the development of Straub phenomena requires an action of the *sacro-coccygeus dorsalis* muscle, and that it is also necessary that the lumbo-sacral cord, with its peripheral nervous outflow, should be intact and that these functioning units should have an adequate circulation (BILBEY; SALEM; GROSSMAN, 1960).

Seizures are the clinical manifestation of an abnormal, excessive, hypersynchronous discharge of a population of cortical neurons (Broomfield et al., 2006). Experimental studies using animal epilepsy models have shown that NMDA, AMPA and kainate agonists induce seizure activity, whereas their antagonists are suppressive modulators (Broomfield et al., 2006). Thus, since glutamate is the main excitatory neurotransmitter at central nervous system (CNS), it is the pivotal agent involved in seizures and other neurodegenerative disorders (Meldrum, 1994; Platt, 2007).

In this work, we sought to investigate the central and peripheral electrophysiological patterns of Jack Bean Urease in rodents, *in vivo* and *in vitro*, as well as the biochemical correlation of cell viability and glutamate release. As far as our knowledge, this is the first work to present the correlation between the EEG activity of Jack Bean Urease with cell viability and the changes of glutamate release. With this on mind, we expected to contribute to the understand of the ureases convulsive-like activities.

2. Materials and Methods

2.1 Animals

Adult male Swiss white mice and adult male Wistar rats were obtained from PUCRS Animal House. The animals were housed in standard rodent cages in a colony room maintained at 24 ºC under a 12 h light/12 h dark cycle with free access to food and water.

The studies presented on this paper have been done in accordance with the Brazilian Council for Animal Experimentation (CONCEA) guidelines and approved by the local animal care committee (CEUA) with the protocol acceptance number 043/2015. Experiments were designed to minimize the number of animals used and their suffering.

2.2 Reagents

All reagents used in these studies were of high purity, obtained from Sigma-Aldrich Co. Brazil.

2.3 Biochemical assays

2.3.1 MTT Cell Viability Assay

2.3.1.1 Hippocampal Slices Preparation

Mice were decapitated, the brains removed immediately, and the hippocampus dissected on ice and humidified in cold HEPES-saline buffer gassed with O_2 (124mm NaCl, 4mM KCl, 1.2mM $MgSO_4$,12mM glucose, 1mM $CaCl_2$, and 25mM HEPES pH 7.4). Hippocampal slices were obtained according to Vinadé and Rodnight (1996), briefly: a Mcilwain tissue chopper was used to obtain the slices (400nm) that were separated and preincubatedat 37 °C for 30min in microwell plates filled with HEPES saline (200 μL/slice). Subsequently, fresh medium was replaced (200 μL/slice) for control condition and treatments with JBU (1µM, 100nM, 10nM, 1nM, 100pM, 10pM and 1pM), and incubated for 1 hour and 30 minutes (37∘C).

2.3.1.2 *Hippocampal Slices Viability*

Immediately after incubation with different treatments, slices were assayed for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (0.05% in HEPES-saline) for 30 min (37°C). The MTT is converted into a purple formazan product after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of DMSO, resulting in a colored compound whose optical density (λ = 490 nm) was measured in an ELISA reader equipment. The MTT colorimetric assay was performed as described by (DAL BELO et al., 2013).

2.3.2 Synaptosomes preparation

Male adult Wistar rats (200–250 g) were sacrificed by decapitation. The brain cortex was removed and homogenized in 0.32 M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (1 mM). Homogenates were then submitted to low-speed centrifugation (1000 g for 10min) and the synaptossomes were purified from the supernatant by

discontinuous Percoll density gradient centrifugation (39,000 g for 15 min) (DUNKLEY; JARVIE; ROBINSON, 2008). The isolated nerve terminals were resuspended in Krebs–Ringer–HEPES (KRH) solution (124 mM NaCl, 4 mM KCl, 1.2 mM Mg2SO4, 10 mM glucose, 25 mM HEPES, pH 7.4) to a final concentration of approximately 1 mg/ml. Aliquots of 30 ml were prepared and kept on ice until use.

2.3.3 Measurement of continuous glutamate release

Glutamate release assessed by measuring the fluorescence increase due to the production of NADPH in the presence of glutamate dehydrogenase and $NADP^+$. This protocol was performed essentially as described by (LOMEO et al., 2014). In short, the medium used for the reaction contained a mixture of rat cortical synaptosomes (± 30 µg of protein/well) and $NADP^+$ (1 mM) in KRH (Krebs Ringer Hepes) was transferred to Elisa microplates (300 µl/well) to be read in a spectrofluorimeter (Synergy 2, Winooski, USA). After 1 min, glutamate dehydrogenase (35 units per well) was added to the medium and the reading was restarted until the fluorescence reached balance (about 10 min). Subsequently, treatments with different concentrations of JBU were applied. Calibration curves were done in parallel by adding known amounts of glutamate to the reaction medium. The experimental data were expressed as nmol of glutamate released per mg of protein. The experiments were performed at 37 ºC for 45 min continuous reading with 360 nm/450 nm excitation/emission wavelengths.

2.4 Electrophysiological recordings

2.4.1- In vitro recordings of compound action potentials from mouse sciatic nerve preparation

Sciatic nerves were obtained from adult male Swiss mice (28 – 35g). The extraction surgery was performed essentially as described by Bala et al. 2014. The mice sciatic nerves

were mounted on a recording chamber according to Dal Belo et al. 2005. Standard extracellular recording techniques were used to record compound nerve action potentials. Pellet-type silver electrodes were dipped into each of the three compartments of the recording chambers, with stimulation occurring between the central and one of the external compartments. Recordings were obtained from the central compartment. A Grass S48 stimulator was used to supply supramaximal electrical impulses (0.4 Hz, 0.04 ms duration) via a model SIU 5A stimulus isolation unit (Grass Instrument Co.). The signals were amplified with a CED1902 transducer (Cambridge Electronic Design, Cambridge, England), digitized with a CED 1401 analogue-to-digital converter (Cambridge Electronic Design) and analyzed with custom built software (Dempster, 1988). In each experiment, the amplitude, rise time, latency and threshold of the action potentials recorded were measured. Prior to add the test compounds, the sciatic nerve preparations were incubated in physiological solution for 15 min under constant supramaximal stimulation to demonstrate viability of the preparation and consistency of the recordings.

2.4.2. In vivo rat electroencephalographic (EEG) Recordings

2.4.2.1. Implantation of animals for electroencephalographic recordings

Animals were anesthetized with ketamine 90 mg/kg and xylazine 13mg/kg. The anesthesia was maintained by supplementary doses of ketamine/xylazine (30% of initial dose), after checking tail pinch reflex and respiratory rate. Body temperature was maintained at 37 ± 0.5 ºC using a heat pad. For the EEG recordings, A-M SYSTEMS .0045" tungsten Teflon-insulated electrodes were employed. These electrodes were surgically implanted on mPFC (3.0 mm anterior to bregma, 0.4 mm lateral to midline and 3.2 mm ventral to dura mater); CA_1 (5.7 mm anterior to bregma,4.6 mm lateral to midline and 2.5 mm ventral to dura mater) and TMD (-1.9 mm anterior to bregma, -0.4 mm to midline and -4.8 mm ventral to

dura mater). The electrode implanting sites coordinates were followed according to Paxinos and Watson (2007). At the hippocampal CA1 area, a chemitrode (electrode + cannula) was implanted. The cannula was used for intrahippocampal injections of 10 nM JBU in a volume of 2 µl. The skull was exposed, and holes were drilled so that the recording electrodes and the chemitrode could be lowered at the mentioned stereotaxic coordinates. An additional hole was drilled over the parietal to cortex to implant a microscrew that served as a recording reference (ground). An additional electrode was used as miogram to detect the electrical activity of the animal's neck muscles in order to detect REM sleep, noise caused by body movements or electrical oscilations. The apparatus employed in the EEG recordings is shown in Fig1.

2.4.2.2. Recording of SWDs

EEG was recorded by an electroencephalograph (NIHONCOHDEN, Japan, Tokyo) attached to a data capture and analysis device (CED: Cambridge Electronic Design Ltd., UK, Cambridge; POWER 1401 mkII). Sampling rate was 2000 Hz whereas the bandwidth of the EEG recording was 0.3–150 Hz. Number of SWDs (frequency: 3–11 Hz; a train of asymmetric spikes and slow waves starting and ending with sharp spikes; the average amplitude at least twice as high as the basal EEG activity) (Kovács et al., 2006, 2015) and time of SWDs (average time and total time of SWDs) were measured between 30 and 270 min of post-injection time (from 4.00 PM to 8.00 PM). Injections and handling induced stress and behavioral alteration may change the SWD number Kovács et al., 2006, 2015). Thus, in spite of that the changes in behavioral features after drug application disappeared within 30 min after injections and normal grooming was observed in all animals, data of the first 30 min after the injections (from 3.30 PM to 4.00 PM) was excluded from the analysis (Kovács et al., 2006). The recording periods were split into 60 min sections, which were evaluated

separately. SWDs were cut off from the raw data files and checked by FFT analysis (Kovács et al., 2006).

EEG recordings were carried out in rats awaked without anesthesia during 6h in a recording chamber containing food and water *ad libitum*. After 2h of basal recordings, the animals were injected with 2µl of JBU 10nM by using a Hamilton syringe connected to a 30G dental needle through a polypropylene flexible pipe. The needle was inserted into the cannula and stood 0,1mm above hippocampal CA_1. Every microliter of JBU solution was injected in one minute.

2.5. Statistical analysis

For the MTT cell viability assay, the results were expressed as the mean ±SEM and were compared statistically using ANOVA for repeated measures. Statistical analysis of the data of glutamate release were made by the method of two-way ANOVA followed by Bonferroni test. For compound action potentials recordings, the results were expressed as the mean ±SEM and were compared statistically, using Student's unpaired *t*-test.

3. Results

3.1 MTT Cell Viability Assay

On MTT Assay no meaningful cell loss could be verified according to our data (Fig. 2). Since MTT is based on mitochondrial dehydrogenases activity, our findings suggest no mitochondrial dysfunctions.

3.2 L-Glutamate Liberation Assay

Rat brain cortex synaptosomes consist of isolated nerve terminals which still contain the machinery related to neurotransmission and represent an excellent model for the study of the effects of substances with potential activity in the central nervous system (CNS). The

analysis of L-Glu release in presence of different concentrations of JBU (1 nM, 10 nM, 20 nM, 50 nM and 100nM), showed a time-dependent significative increase in the release of L-GLU (n=6) Fig.3. Thus, 33.34±8.56% maximum increase of JBU-induced L-Glu release was observed with the concentration of 100nM in 45min readings ($p<0.05$) Table 1. Also, a positive control in depolarizing conditions (KCl) was assayed for comparison.

3.3 Compound action potential (CAP)

The effect of JBU on compound action potentials was limited to significant changes in the amplitude and rise time of the potentials. Fig. 4 displays JBU's effect in different doses. This result is summarized in Table 2.When 0.001 µM JBU was applied, the most significant decrease (40) in CAP amplitude was observed ($n = 6$, $p <0.05$). The subsequent application of Tetrodoxin (0.1µM) when applied after or before JBU in the preparation did not increase or decrease the urease effect (Fig.5).

3.4 Electroencephalographic (EEG) Recordings

An intrahippocampal injection of JBU 10 nM, induced a 3 Hz generalized spike-wave discharge as shown in Fig. 6. This suggests a non-convulsive seizure, a petit mal seizure, since the animals did not respond to external sound stimulation or show any tonic clonic convulsion.

3.4.1 Effects of Jack Bean Urease on mean number of SWDs

The intrusion criteria for SWDs in this study were that its duration should be more than 3 seconds. At the end of recording we calculated numbers of SWDs that were more than 3 seconds. Numbers of SWDs in all groups are presented in Fig.7. There was a noticeable number of SWDs in all tested areas, slightly higher but not statistically meaningful in CA_1.

3. Discussion

In this work we have shown that intrahippocampal injections of rats with Jack Bean Urease provoked spike-and-wave episodes related to absence crisis phenomena. In addition, it was demonstrated that the induction of such electrophysiological deregulations are accompanied by an increase of L-GLU release, without affecting the neuronal viability, as shown by the MTT assay in mice. The inability of TTX to induce a further inhibitory activity on the onset of JBU blockage of mouse sciatic nerve conduction, suggests that the urease displays an activity over voltage-gated sodium channels. The results will then be discussed in detail herein.

Spike-and-wave is the term that describes a particular pattern of the electro encephalogram (EEG) typically observed during epileptic seizures. The spike-and-wave discharge is a regular, symmetrical, generalized EEG pattern seen, especially during, absence epilepsy, also known as 'petit mal' epilepsy (AKMAN et al., 2010). The basic mechanisms underlying these patterns are complex and involve part of the cerebral cortex, the thalamocortical network, and intrinsic neuronal mechanisms (SNEAD, 1995). Some studies suggest that a thalamocortical (TC) loop is involved in the initiation of spike-and-wave oscillations (AKMAN et al., 2010; SNEAD, 1995).

Recent studies of thalamic brain slices, suggest that inhibition of thalamic relay neurons by GABA-ergic interneurons hyperpolarizes the relay neuron, thus removing inactivation of the T-type Ca^{2+} channels (MCCORMICK; CONTRERAS, 2001). This sequence of events leads to a rebound burst of action potentials after each inhibitory postsynaptic potentials (IPSP). The action potential stimulates the GABAergic neurons by a reciprocal excitatory connection. The action potentials in the relay neurons also excite cortical neurons and thus can be manifested in the EEG as a "spindle" (MCCORMICK; CONTRERAS, 2001).

Block of $GABA_A$ channels enhances $GABA_B$ IPSPs in relay neurons, resulting in an increase in rebound bursts of action potentials. Thus the T-type Ca^{2+} channel and $GABA_B$ receptors appear to play an important role in generating activity in mammalian absence seizures (CAIN; SNUTCH, 2012; CHEN; PARKER; WANG, 2014; IFTINCA, 2011).

In our experimental conditions JBU was injected directly at the rat hippocampus, where the number of SWDs was slightly higher compared to other brain regions, although not significative. This latter result suggests that JBU induces intense hypersynchronic bursts firing activity on CA1 area that turned the electrical activity to spread to other brain regions. Indeed, in a recent study we have shown that JBU is increasing Ca^{2+} influx in primary hippocampal cultures (Piovesan et al., 2015). Although we have not addressed which type of calcium channel is involved in the JBU-increase of calcium influx in hippocampus, there is a striking link between the involvement of T-type calcium channels at CA1 area of hippocampus and the development of SWDs (Iftinca, 2011).

In this regard, Bengtson et al., (2013) have shown that in a twelve-day hippocampal culture, neurons normally express markers for either glutamate (~ 90% of neurons) or GABA (~ 10% of neurons). As described above, the mechanisms involved in absence-like crises are complex. The specific cellular mechanisms by which absence seizures are produced are still a matter of debate, including the extent of involvement of GABAergic signaling (Cope et al., 2009). In this view, in our experimental conditions two situations could be amenable to explain the pharmacological activity of JBU over mammalian nervous system: the former and less reasonable, is that the blocking activity of JBU on sodium channels, as seen with the decrease of mouse CAP conductance, would involve specific gabaergic neurons, similarly to the central actions of local anesthetics. This pharmacological interaction with voltage-gated sodium channels would cause a relatively selective depression of inhibitory neurons, which ultimately would induce cerebral excitation, therefore provoking seizures (HARA; KAI;

IKEMOTO, 1995). The second and more attractive possibility appears to be an increase of gabaergic transmission by the blockage of voltage-gated sodium channels in glutamatergic neurons, since these excitatory cells are more abundant in CNS. In this context, increased GABAergic transmission may predominantly represent a compensatory response of the brain in an attempt to decrease seizure propensity. However, enhanced GABA transmisson has been reported paradoxically to promote seizures as well, at least in some specific types of epilepsy models (Klaasen et al., 2006), such as absence epilepsy (Cope et al., 2009). However, how to explain the JBU-increase of L-Glu release in brain synaptossomes? The explanation may relay on the clinical observation that several antiepileptic drugs such as: phenobarbital, benzodiazepines, phenytoin, carbamazepine, oxcarbazepine, valproate, ethosuximide among others can exacerbate seizures, in which glutamate content is positively altered (OTOOM; AL-HADIDI, 2000).

Finally, the inability of JBU to decrease the neuronal cell viability may suggests that the amount of glutamate release during the onset of the toxin activity was not harmful to the cells. Indeed a similar activity was observed by with pancreatic islets (Barja-Fidalgo et al., 1991) and platelets (Carlini et al., 1985). This later result permit to infer that mitochondrial dysfunctions are not associated with the JBU-induced seizure activity (Henshall 2007; Zsurka and Kunz 2015).

Acknowledgments

The authors thank the Coodenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES Edital Toxinologia 063/2010. Carlos Gabriel Moreira de Almeida was granted by CAPES fellowship. The authors declare no conflicts of interest related to this work.

4. References

AKMAN, O. et al. Electroencephalographic differences between WAG/Rij and GAERS rat models of absence epilepsy. **Epilepsy Research**, v. 89, n. 2-3, p. 185–193, 2010.

BALA, U. et al. Harvesting the maximum length of sciatic nerve from adult mice: a step-by-step approach. **BMC Research Notes**, v. 7, n. 1, p. 714, 2014.

BECKER-RITT, A. B. et al. Antifungal activity of plant and bacterial ureases. **Toxicon**, v. 50, n. 7, p. 971–983, 2007.

BENGTSON, C. P. et al. Calcium responses to synaptically activated bursts of action potentials and their synapse-independent replay in cultured networks of hippocampal neurons. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1833, n. 7, p. 1672–1679, 2013.

BILBEY, D. L. J.; SALEM, H.; GROSSMAN, M. H. The anatomical basis of the straub phenomenon. **Brit J Pharmacol**, v. 15, p. 540–543, 1960.

CAIN, S. M.; SNUTCH, T. P. Voltage-gated calcium channels in epilepsy. **Jasper' s Basic Mechanisms of the Epilepsies**, 2012.

CARLINI, C. R.; POLACCO, J. C. Toxic Properties of Urease. **Crop Science**, v. 48, n. 5, p. 1665, 2008.

CHEN, Y.; PARKER, W. D.; WANG, K. The role of T-type calcium channel genes in absence seizures. **Frontiers in Neurology**, v. 5 MAY, n. May, p. 1–8, 2014.

DAL BELO, C. A. et al. Pharmacological and structural characterization of a novel phospholipase A2 from Micrurus dumerilii carinicauda venom. **Toxicon**, v. 46, n. 7, p. 736–750, 2005.

DAL BELO, C. A. et al. In Vitro Antiophidian Mechanisms of Hypericum brasiliense Choisy Standardized Extract: Quercetin-Dependent Neuroprotection. **BioMed Research International**, v. 2013, p. 1–6, 2013.

DIXON, N. E. et al. Letter: Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? **Journal of the American Chemical Society**, v. 97, p. 4131–4133, 1975.

DUNKLEY, P. R.; JARVIE, P. E.; ROBINSON, P. J. A rapid Percoll gradient procedure for preparation of synaptosomes. **Nature protocols**, v. 3, n. 11, p. 1718–1728, 2008.

FERREIRA-DASILVA, C. T. et al. Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. **Archives of insect biochemistry and physiology**, v. 44, n. April, p. 162–171, 2000.

FOLLMER, C. et al. Jackbean, soybean and Bacillus pasteurii ureases: Biological effects unrelated to ureolytic activity. **European Journal of Biochemistry**, v. 271, n. 7, p. 1357–1363, 2004.

HARA, M.; KAI, Y.; IKEMOTO, Y. Local anesthetics reduce the inhibitory neurotransmitter-induced current in dissociated hippocampal neurons of the rat. **European Journal of Pharmacology**, v. 283, n. 1-3, p. 83–89, 1995.

HENSHALL, D. C. Apoptosis signalling pathways in seizure-induced neuronal death and epilepsy. **Biochemical Society transactions**, v. 35, n. Pt 2, p. 421–423, 2007.

IFTINCA, M. C. Neuronal T-type calcium channels: what's new? Iftinca: T-type channel regulation. **Journal of medicine and life**, v. 4, n. 2, p. 126–138, 2011.

KRAJEWSKA, B. Ureases I. Functional, catalytic and kinetic properties: A review. **Journal of Molecular Catalysis B: Enzymatic**, v. 59, n. 1-3, p. 9–21, 2009.

LOMEO, R. DA S. et al. Crotoxin from Crotalus durissus terrificus snake venom induces the release of glutamate from cerebrocortical synaptosomes via N and P/Q calcium channels. **Toxicon**, v. 85, p. 5–16, 2014.

MARTINELLI, A. H. S. et al. Structure-function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (Canavalia ensiformis) urease. **Biochimica et Biophysica Acta - General Subjects**, v. 1840, n. 3, p. 935–944, 2014.

MCCORMICK, D. A.; CONTRERAS, D. O N T HE C ELLULAR AND N ETWORK B ASES. n. 1, 2001.

MOBLEY, H. L.; ISLAND, M. D.; HAUSINGER, R. P. Molecular biology of microbial ureases. **Microbiological reviews**, v. 59, n. 3, p. 451–480, 1995.

OTOOM, S.; AL-HADIDI, H. Seizure induced by antiepileptic drugs. **Annals of Saudi Medicine**, v. 20, n. 3-4, p. 316–318, 2000.

PLATT, S. R. The role of glutamate in central nervous system health and disease - A review. **Veterinary Journal**, v. 173, n. 2, p. 278–286, 2007.

SNEAD, O. C. Basic mechanisms of generalized absence seizures. **Annals of Neurology**, v. 37, n. 2, p. 146–157, 1995.

VINADÉ, L.; RODNIGHT, R. The Dephosphorylation of Glial Fibrillary Acidic Protein (GFAP) in the Immature Rat Hippocampus is Catalyzed Mainly by a Type 1 Protein Phosphatase. **Brain research**, v. 732, p. 195–200, 1996.

WONG, M. Too much inhibition leads to excitation in absence epilepsy. **Epilepsy currents / American Epilepsy Society**, v. 10, n. 5, p. 131–132, 2010.

ZSURKA, G.; KUNZ, W. S. Mitochondrial dysfunction and seizures: the neuronal energy crisis. **The Lancet Neurology**, v. 14, n. 9, p. 956–966, 2015.

5. Figures

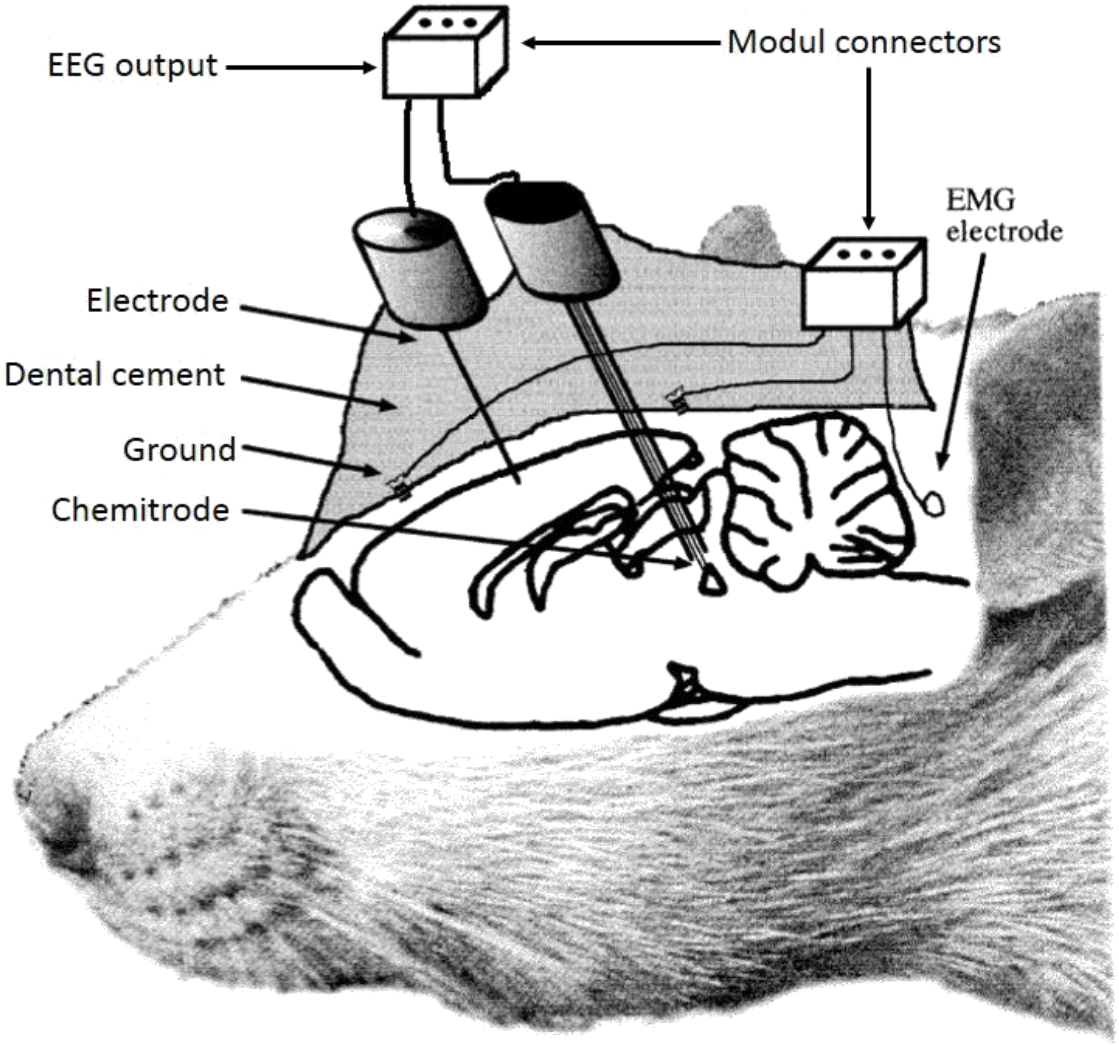


Figure 1- EEG Apparatus

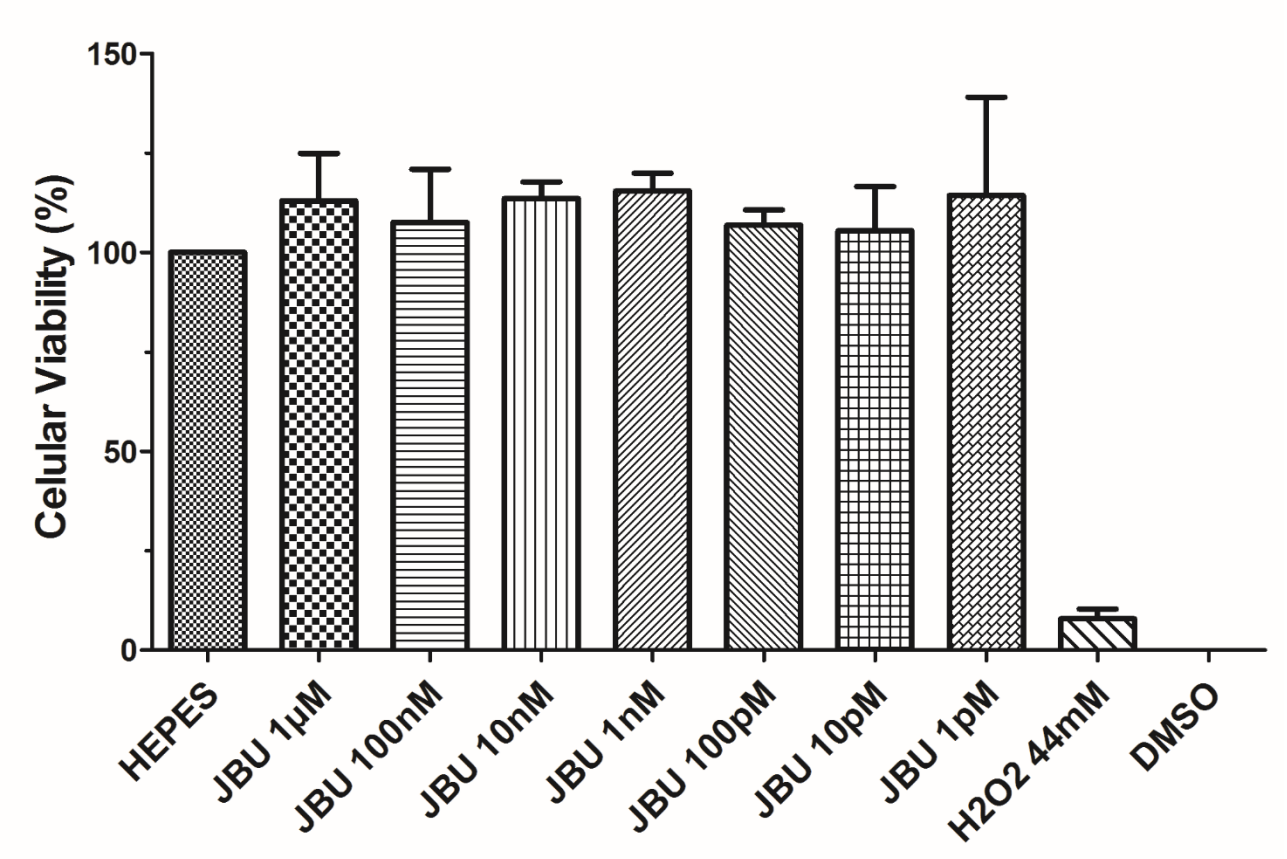


Figure 2- MTT Cell Viability Assay

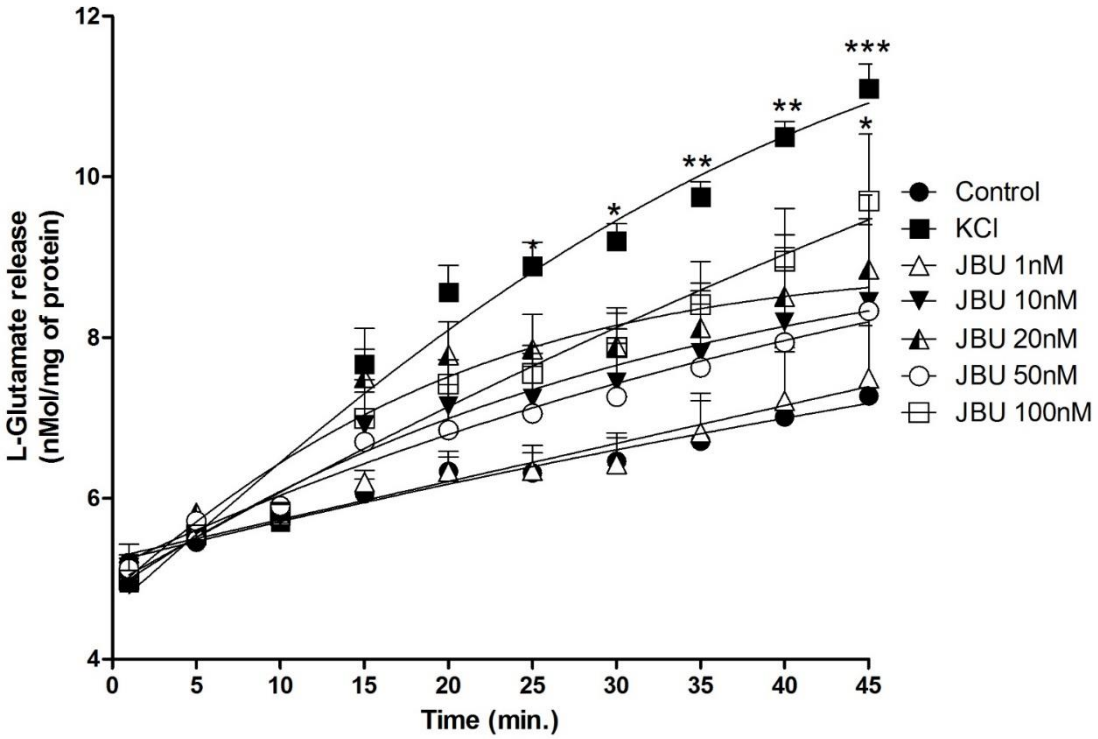
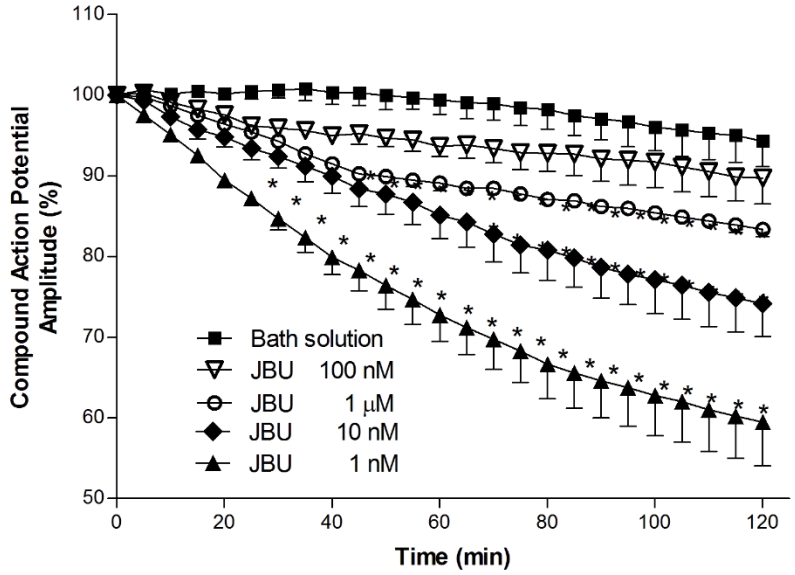


Figure 3 – L-Glu release

A



B

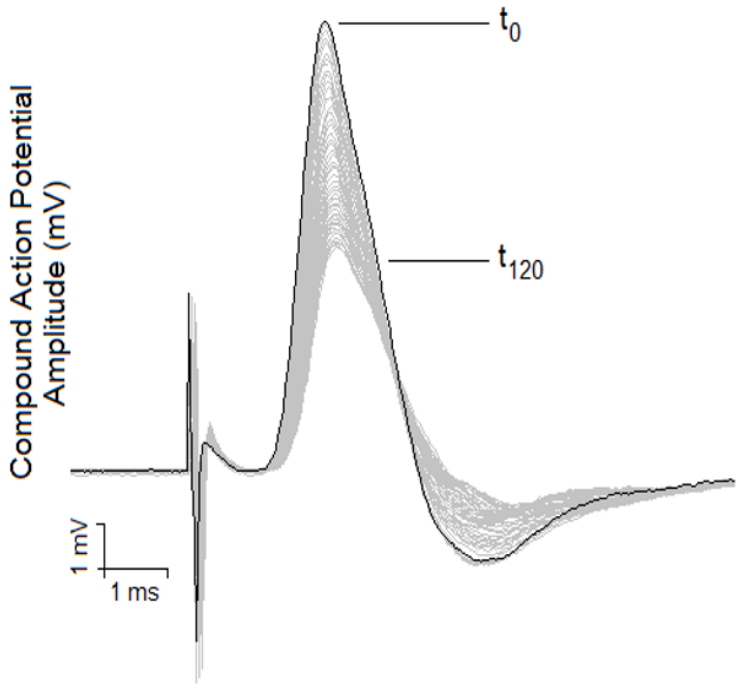


Figure 4 – Compound Action Potential

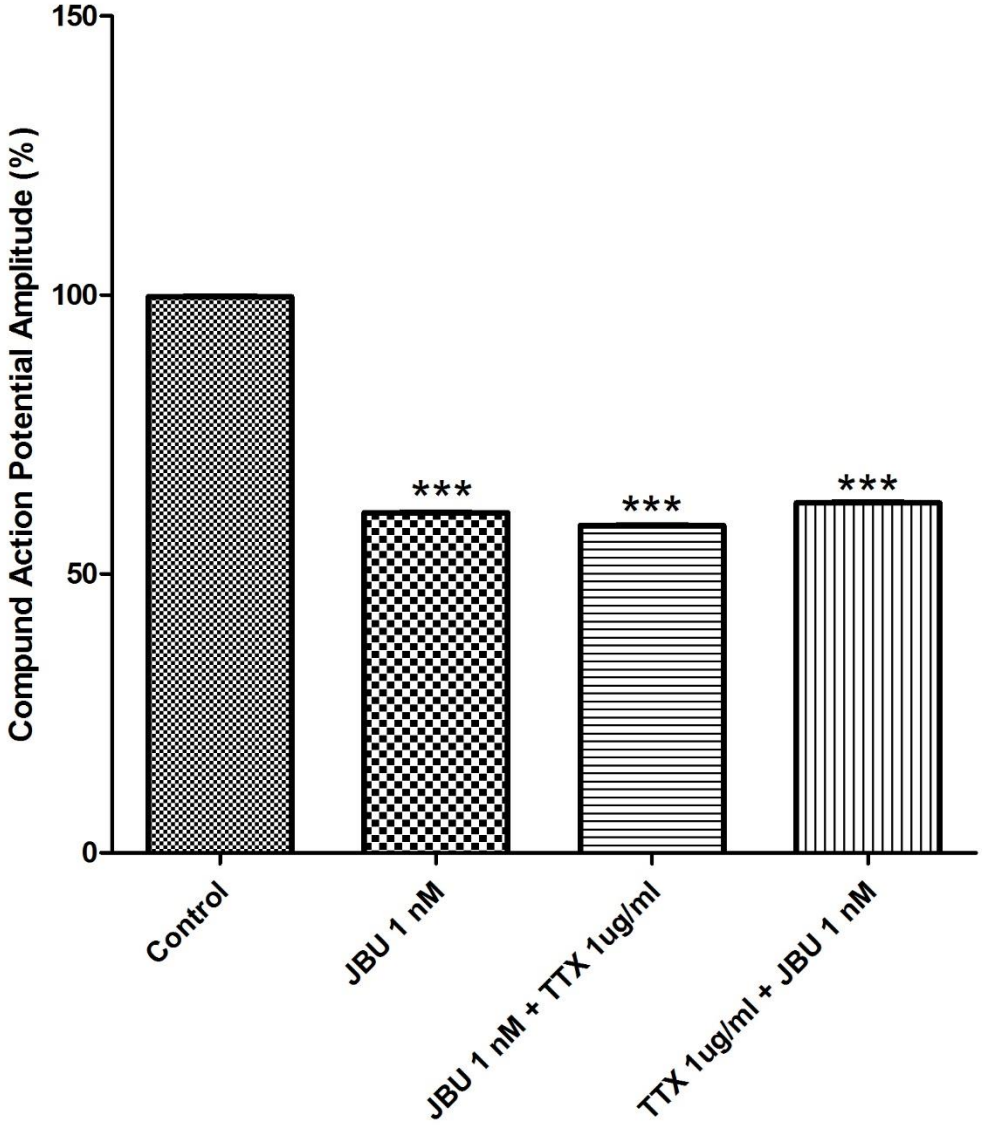


Figure 5 – Compound Action Potential Amplitude.

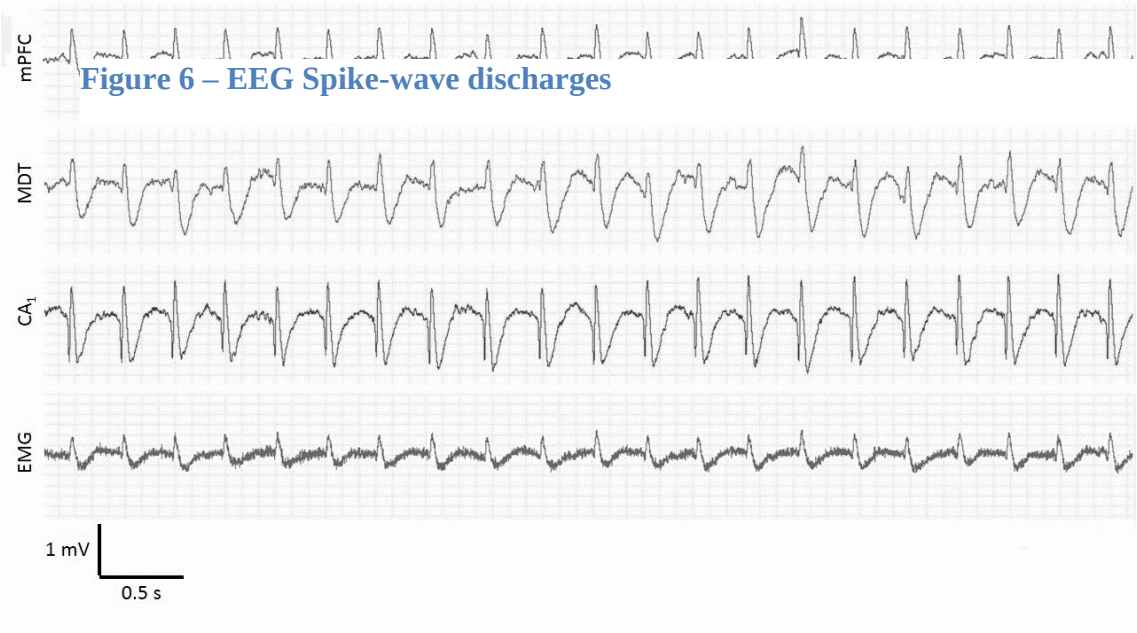


Figure 6 – EEG Spike-wave discharges

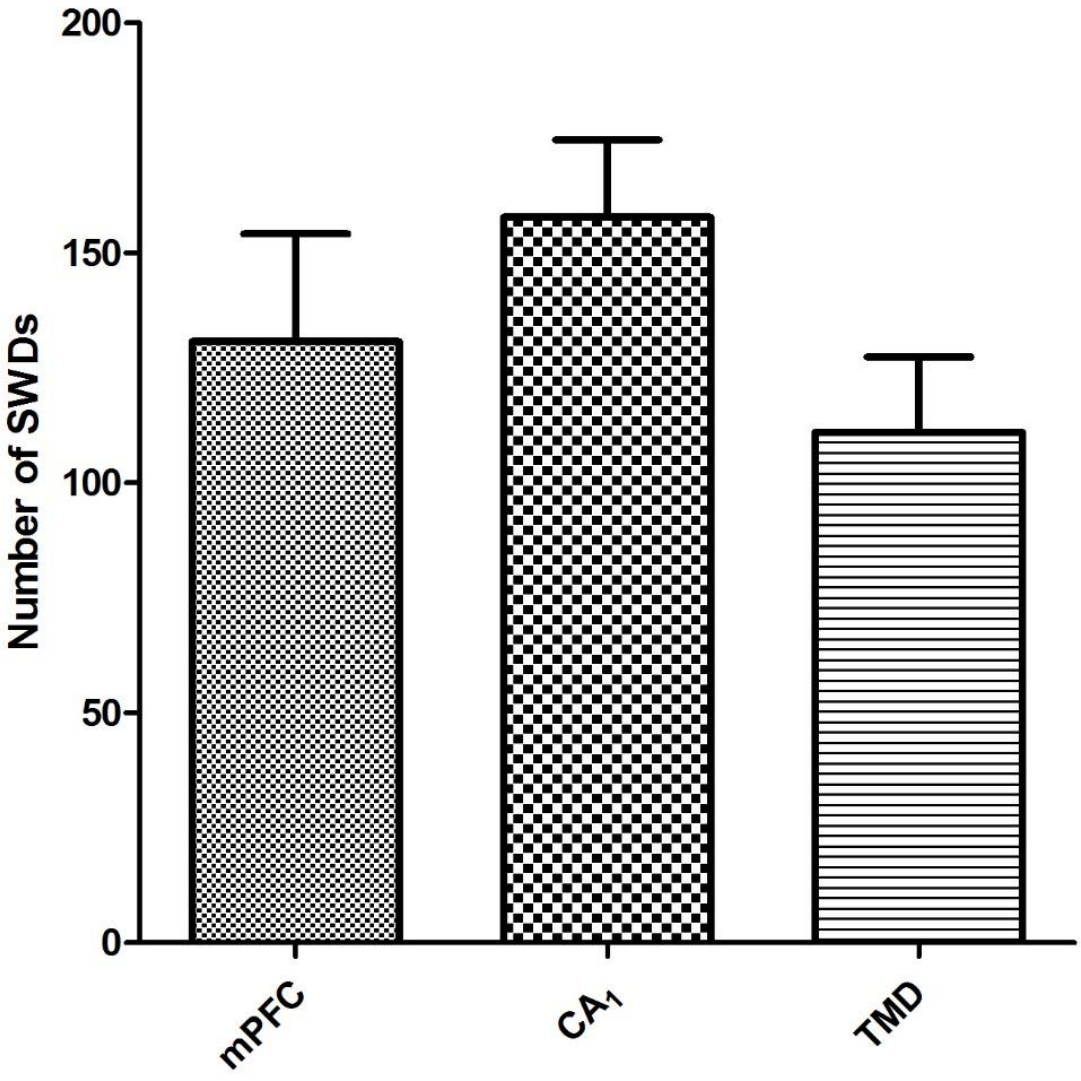


Figure 7 – Number of SWDs

6. Tables

Table 1

Electrophysiological parameters recorded from the mouse sciatic nerve pre-incubated with different concentrations of JBU.

Treatment	Amplitude (mV)		Latency (ms)		Rise Time (ms)	
	Time 0 (absolute values)	End of incubation (% time 0)	Time 0 (absolute values)	End of incubation (% time 0)	Time 0 (absolute values)	End of incubation (% time 0)
Bath solution	8.0±0.6	94.3±3.2	0.26±0.06	122±8.8	0.25±0.03	90.1±2.9
JBU 1 µM	8.9±2.5	83.4±0.9*	0.35±0.10	114±1.8	0.25±0.02	93.7±4.3
JBU 100 nM	10.8±2.9	74.2±4.1*	0.44±0.05	106±2.4	0.26±0.03	89.3±8.8
JBU 10 nM	12.9±2.9	89.7±2.9	0.59±0.11	105±0.9	0.33±0.08	94.5±5.8
JBU 1 nM	7.7±3.7	59.5±5.4*	0.55±0.06	107±3.6	0.28±0.01	91.5±3.9

*$p<0.05$ compared to t_0 values in all cases.

Table 2

Average percentage of L-glutamate release increase of all treatments compared to control.

Treatment	Increase in L-Glutamate release (%)
Control	0
KCl	52.55 ± 2.7*
JBU 1 nM	3.08 ± 10.4
JBU 10 nM	16.08 ± 12.25
JBU 20 nM	21.65 ± 10.43
JBU 50 nM	14.56 ± 12.9
JBU 100 nM	33.34 ± 8.56*

*$p<0.05$ compared to t_0 values in all cases.