UNIVERSIDADE FEDERAL DO PAMPA

***CAMPUS* SÃO GABRIEL**

PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM CIÊNCIAS BIOLÓGICAS

ANA PAULA DE BAIRROS LUCHO

EFEITOS DO RILUZOLE NO SISTEMA NERVOSO CENTRAL E PERIFÉRICO DE VERTEBRADOS

SÃO GABRIEL, RIO GRANDE DO SUL, BRASIL,

2014

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

Orientador: Profª. Drª. Lúcia Vinadé.

Co-Orientador: Prof. Dr. Chárist on André Dal Belo.

São Gabriel,

2014

LUCHO, Ana Paula de Bairros

Efeitos do riluzole no sistema nervoso central e periférico de vertebrados / Ana Paula de Bairros Lucho.

57 folhas;

Dissertação (Mestrado) Universidade Federal do Pampa, 2014.
Orientação: Profª. Drª. Lúcia Vinadé.

1. Neurobiologia. 2. Farmacologia. 3. Bioquímica
I. Vinadé, Lúcia. II. Doutor

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Área de Concentração: Ciências Biológicas

Linha de Pesquisa: Qualidade Ambiental

Dissertação defendida e aprovada em: 15/05/2014.
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A minha orientadora, professora e exemplo de profissional, Lúcia Vinadé.

AGRADECIMENTOS

A Profª. Dra. Lúcia Vinadé, desejo mais que agradecê-la. Queria poder manifestar em palavras a admiração e respeito que tenho por sua pessoa. Muito obrigada pela oportunidade de trabalhar ao seu lado, mais do que tudo, pela confiança em mim depositada. Resta-me apenas dizer, que tenho orgulho de ser sua aluna. Obrigada.

Ao Prof. Dr. Chárist on Dal Belo, pelos ensinamentos ao longo deste trabalho. Pela sabedoria nos momentos de dúvidas e por se fazer presente mesmo estando longe. Muita Obrigada por tudo!

Ao pessoal da Citogenética, Thiago, Leti, Vanusa e Thais e a Profª. Drª. Analia, pela disponibilidade em ajudar.

Ao Rafa K. pela amizade que se iniciou, pela ajuda quando necessário e por me acompanhar no lab até tarde da noite.

A Ana Paula Perin pela ajuda e paciência neste trabalho, e comigo.

A Grazi Stürmer pela amizade ao longo da Graduação que se fez continua.

Aos Profs. Lisiane Porciuncula e Fábio Klant, pela oportunidade em seus respectivos laboratórios, na UFRGS.

Aos Profs. Thais Posser e Jeferson Franco e a MSc. Ana Paula Zemolin do Laboratório de Sinalização Celular – UNIPAMPA, pela disponibilidade sempre.

Ao pessoal do Laboratório de Neurobiologia e Toxinologia (LANETOX): Mari, Darlene, Mayra e Jhulimar, pela ajuda de forma direta ou indireta neste trabalho.

A Malu pela amizade e apoio nos momentos de e tristeza e choro.

Ao Francisco pelo amor e principalmente paciência comigo, nestes 24 meses de Mestrado!

Aos meus Pais, espero fazê-los sentirem-se orgulhosos hoje e sempre. Amo vocês!

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.

William Ernest Henley

RESUMO

O Riluzole é quimicamente relacionado aos benzotiazóis e é conhecido como um agente neuroprotetor, possuindo propriedades anticonvulsivantes, analgésicas, anestésicas, e sedativas. A ação neuroprotetora mais conhecida desta droga ocorre através da inibição da transmissão glutamatérgica no sistema nervoso central (SNC). Nesse trabalho, o Riluzole foi ensaiado sobre a junção neuromuscular esquelética (JNM) de aves visando estudar sua interação com o sistema nervoso periférico (SNP). Também foi verificada a ação do Riluzole em fatias de hipocampo de camundongos, comparando os resultados com agentes anti-inflamatórios como o extrato de *Hypericum brasiliense* (HBE) e seu principal composto, quercetina, frente ao veneno de serpente *Crotalus durissus terrificus* (Cdt). No SNP, o Riluzole foi ensaiado em preparação músculo biventer cervicis de pintainhos, em banho de órgão isolado, nas doses de 5, 10 e 20 µM. Foram obtidos registros da amplitude da força de contração muscular em presença ou ausência de Riluzole durante 120min, e as curvas-resposta à adição exógena de acetilcolina (ACh – 110 µM) e ao cloreto de potássio (KCl – 20 mM), antes e após a incubação com o tratamento. O Riluzole induziu respostas tempo e dose-dependentes. Na concentração de 5 µM houve uma diminuição gradativa e significativa da resposta contrátil ($p<0.05$). Na concentração de 10 µM, houve uma facilitação significativa ($p<0.05$) da resposta contrátil e das curvas evocadas pelo KCl e ACh. No entanto, na dose de 20 µM houve uma estabilização da contratilidade em relação ao controle. Em todas as doses de Riluzole ensaiadas na JNM houve um aumento significativo da atividade da enzima acetilcolinesterase (AChE). Na sequência da verificação do mecanismo de ação do Riluzole sobre a placa motora, registros eletromiograficos foram tomados na presença dos inibidores especificos, Neostigmina e d-Tubocurarina, onde foi observada uma reversão dos efeitos quando Riluzole foi adicionado ao meio. Como modelo celular de SNC, a ação do Riluzole foi ensaiada em fatias de hipocampo e a viabilidade destas frente ao veneno de Cdt foi observada por meio da atividade de desidrogenases mitocondriais. Tanto Riluzole, como o extrato da planta HBE e quercetina, aumentaram a viabilidade celular em 1h de incubação a 37˚C na presença do veneno. Quercetina foi mais efetiva do que Riluzole e HBE em neutralizar a lise celular induzida pelo veneno. Assim, estes resultados demonstram a influência do Riluzole no SNC como neuroprotetor de toxinas de veneno de serpente, possivelmente atuando como agente anti-inflamatório, e no SNP, aumentando a atividade da AChE e atuando de maneira dose-dependente sobre a placa motora.

Palavras-Chave: junção neuromuscular, veneno, quercetina, acetilcolina, biventer cervicis.

ABSTRACT

Riluzole is chemically related to benzothiazoles and it is known as a neuroprotective agent with anticonvulsant, analgesic, anesthetic and sedative properties. The neuroprotective drug action is well established being through inhibition of glutamatergic transmission in the central nervous system (CNS). In this study, we have assayed the drug Riluzole at skeletal neuromuscular junction (NMJ) of avian, seeking its interaction with the peripheral nervous system (PNS). We also tested Riluzole in the CNS of mice by comparing its results with anti - inflammatory agents, such as extract of *Hypericum brasiliense* (HBE) and its main isolated compound, quercetin, against the poison of *Crotalus durissus terrificus* (Cdt). In the PNS, Riluzole was tested in nerve-muscle preparations in chick biventer cervicis at doses of 5, 10 and 20 μM. It was obtained recordings of the muscle twitch-tension amplitude and the contracture responses to exogenous applied acetylcholine (ACh 110 μM) and potassium chloride (KCl – 20 mM) in the presence or absence of Riluzole during 120 min. Riluzole induced time and dose-dependent responses. At concentration of 5μM there was a gradual and significant decrease in the contractile response ($p<0.05$). The concentration of 10μM showed a significant facilitation of the contractile response ($p<0.05$) and an increase in the curve responses evoked by KCl and ACh. However, at a dose of 20 μM there was a stabilization of contractility compared to control. All tested doses of Riluzole showed a significant increase in the activity of the enzyme acetylcholinesterase (AChE). The action mechanism of Riluzole on the endplate was further analysed through the use of specific inhibitors, Neostigmine and d-Tubocurarine, a reversal of effects those was seen when Riluzole was added to the medium. As a cellular model of CNS, the action of Riluzole was tested in mice hippocampal slices. The cell viability against Cdt venom was observed through the activity of mitochondrial dehydrogenases. Riluzole as much as the plant extract HBE and its active ingredient, quercetin, increased cell viability in 1h incubation at 37˚C in the presence of the poison. However, quercetin showed to be more effective than Riluzole and HBE in neutralizing cell lysis induced by the venom. Thus, these results demonstrate the influence of Riluzole on the CNS, it showed a neuroprotective effect against snake venom toxins, possibly acting as an anti-inflammatory agent. As for the cholinergic system in the NMJ, Riluzole showed an interesting effect by increasing the activity of AChE and by acting in a dose-dependent manner over the endplate.

Keywords: neuromuscular junction, poison, quercetin acetylcholine, biventer cervicis.

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LISTA DE ABREVIATURAS E SIGLAS

AcCoA – Acetil - coenzima A

ACh – Acetilcolina (Acetylcholine)

AChE – Acetilcolinesterase (Acetylcholinesterase)

AChRs – Receptores de acetilcolina (Acetylcholine Receptors)

CAT – Colina acetil-transferase

Cdt – *Crotalus durissus terrificus*

CoA – Coenzima A

ELA - Esclerose lateral amiotrófica

GLU – Glutamato

HBE – *Hypericum brasiliense*

HEK – Human embrionic kidney

JNM/NMJ – Junção neuromuscular (Neuromuscular junction)

KCl - Cloreto de potássio

MBC – Músculo biventer cervicis

mM – Milimolar

NEO – Neostigmina

nm - Nanômetro

PLA 2 - Fosfolipase A_2 (Phospholipase A2)

SNAP-25 - Proteína associada à vesícula sináptica (Synaptosomal-associated protein 25)

SNC/CNS - Sistema nervoso central (Central nervous system)

SNP/PNS - Sistema nervoso periférico (Periferic nervous system)

µM – Micromolar

APRESENTAÇÃO

Esta dissertação contempla dois CAPÍTULOS, cada um referente a um manuscrito. Cada capítulo contém uma breve INTRODUÇÃO, os OBJETIVOS, o MANUSCRITO propriamente dito e as CONSIDERAÇÕES FINAIS.

A metodologia realizada e os resultados obtidos nesta dissertação são apresentados nos itens MANUSCRITO I e II, pois nos mesmos constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

As REFERÊNCIAS referem-se somente às citações que aparecem nos itens de introdução desta dissertação.

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CAPÍTULO I

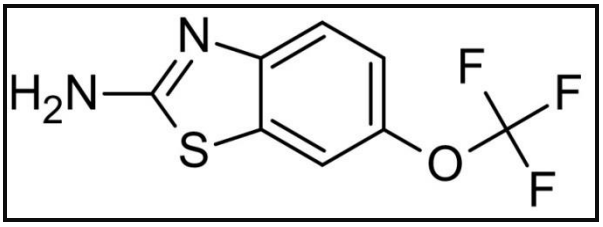
Efeito do Riluzole no Sistema Nervoso Periférico em Preparação Nervo-Músculo Biventer Cervicis de Pintainho

1 INTRODUÇÃO

1.1 Riluzole

O Riluzole, cuja fórmula química é descrita como $C_8H_5F_3N_2OS$, é atualmente o único fármaco aprovado clinicamente para o tratamento de Esclerose Lateral Amiotrófica (ELA) (Figura 1). É um agente neuroprotetor de neurônios motores e prolonga a sobrevida de pacientes com ELA. Este efeito é acreditado como sendo o resultado da modulação na neurotransmissão glutamatérgica nos terminais pré e pós-sinápticos reduzindo, assim, a excitotoxicidade do glutamato (GLU). Esta droga possui várias atividades farmacológicas já descritas como analgésico, anestésico, anti-isquêmico e sedativo (CODERRE *et al.*, 2007; DOBLE, 1996, PRATT *et al.*, 1992). A habilidade para neutralizar pelo menos alguns sintomas de neurodegeneração levou a estudos do Riluzole como um agente contra uma série de doenças do sistema nervoso que afetem as redes motoras (CIFRA *et al.*, 2013).

Figura 1- Fórmula Estrutural do Riluzole.



Fonte: http://en.wikipedia.org/wiki/Riluzole

Vários estudos sugerem que o Riluzole inibe a liberação de aminoácidos excitatórios de tecidos cerebrais, uma vez que diminuí as convulsões evocadas por GLU, reduz a liberação espontânea e deprime a excitação de motoneurônios induzida por agonistas de GLU (revisado em BELLINGHAM, 2011).

A ideia de que o mecanismo de ação do Riluzole pode envolver o bloqueio da transmissão glutamatérgica veio originalmente de estudos neurofarmacológicos *in vivo* do perfil anticonvulsivante desta droga (MIZOULE *et al.*, 1985).O Riluzole revelou-se eficaz em modelos animais de doenças de Parkinson (CARBONE *et al.*, 2012), Doença de Huntington (MARY *et al.*, 1995) e isquemia cerebral e da retina (MALGOURIS *et al*., 1989; LAGREZE *et al*., 1999). Da mesma forma, para estudar e explorar a ação do Riluzole como agente

protetor do neurônio motor, vários modelos experimentais têm sido implementados para mimetizar os processos básicos subjacentes de doenças do neurônio motor e de lesões de medula (CIFRA *et al*., 2011; MAZZONE & NISTRI 2011; ROTHSTEIN & KUNCL 1995).

A excitotoxicidade é um processo patológico em que os neurônios morrem pelo excesso da ativação de receptores glutamatérgico na fenda sináptica causada por altos níveis de GLU, e é fortemente implicada como um fator importante que contribui na patogênese da morte neuronal. A excitotoxicidade glutamatérgica está envolvida em alterações neurológicas agudas como hipóxia, isquemia, traumatismo craniano e epilepsia e em doenças neurodegenerativas (LIPTON & ROSENBERG, 1994).

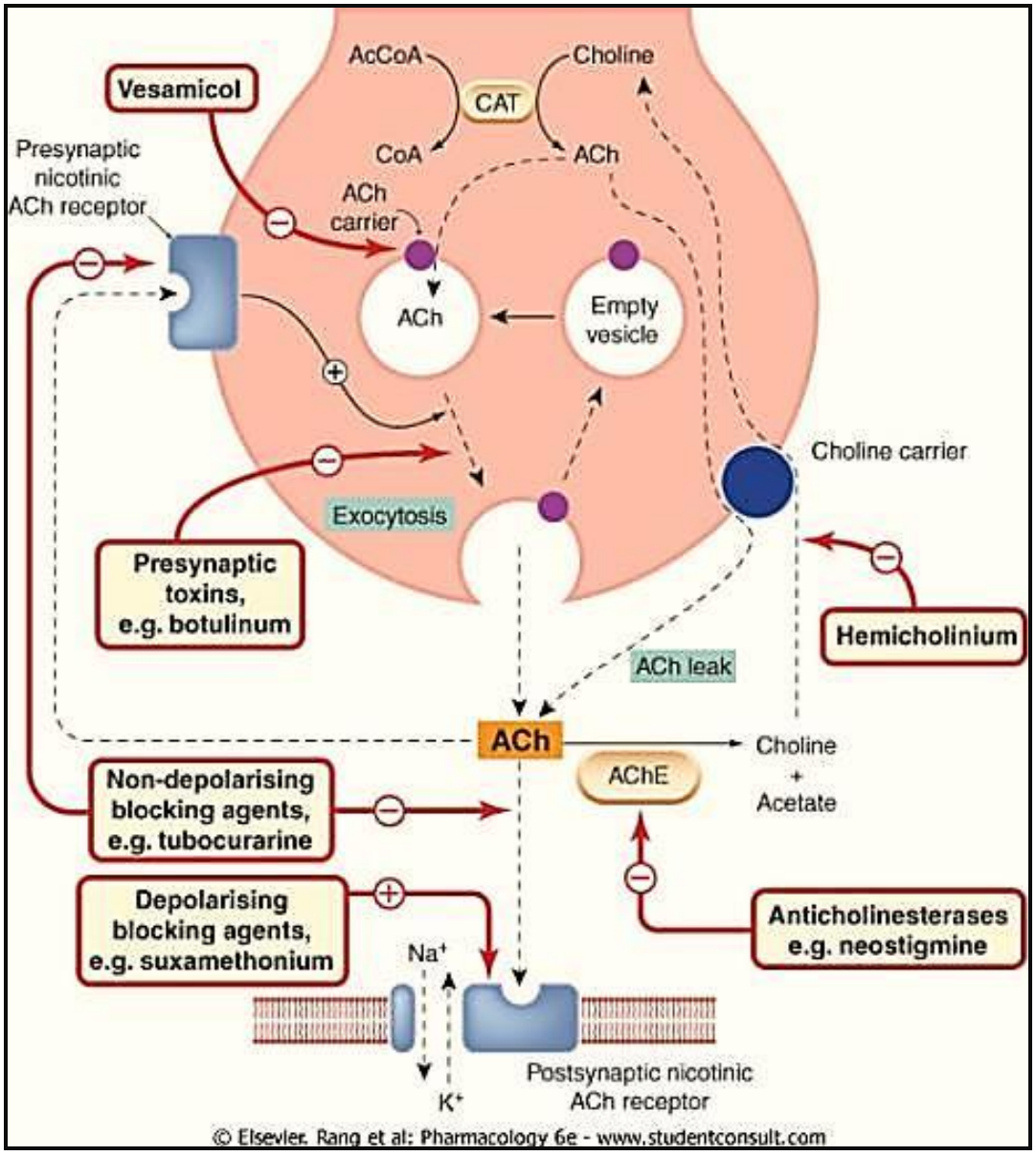
1.2 Junção Neuromuscular

A junção neuromuscular (JNM) é uma estrutura complexa que serve para comunicar de forma eficiente o impulso elétrico dos neurônios motores com o músculo esquelético para sinalizar a contração muscular. As características moleculares e da arquitetura da JNM estão focadas na comunicação entre nervo e músculo. (HUGHES *et al.*, 2006).

A JNM é uma sinapse química, que se diferencia das sinapses centrais por ser apenas excitatória, liberando o neurotransmissor acetilcolina (ACh) que ativa os receptores nicotínicos (AChRs), na região pós-sinaptica. O mecanismo da liberação sináptica (Figura 2) envolve alterações conformacionais das vesículas e diferentes estágios como o docking (aproximação da membrana terminal) e priming (aumento da sensibilidade ao Ca^{2+}) (HUGHES, *et al.*, 2006). Esses dois processos culminam com a exocitose do conteúdo da vesícula na fenda sináptica e dependem de proteínas determinantes como a sintaxina-1 e a proteína associada à vesícula sináptica, SNAP-25, no terminal pré-sináptico (MURTHY & DE CAMILLI, 2003).

A fenda sináptica é um espaço de cerca 50 nm que separa o nervo e a membrana plasmática do músculo esquelético. Os AChRs são as proteínas mais estudadas sob o ponto de vista funcional na JNM. Além disso, têm sido objeto de inúmeras investigações com o intuito de aprofundar o conhecimento sobre a sua relação com alvos antigênicos primários e doenças neurodegenerativas do terminal nervoso motor (HUGHES *et al.*, 2006).

Figura 2- Ilustração do Mecanismo de Liberação de Acetilcolina



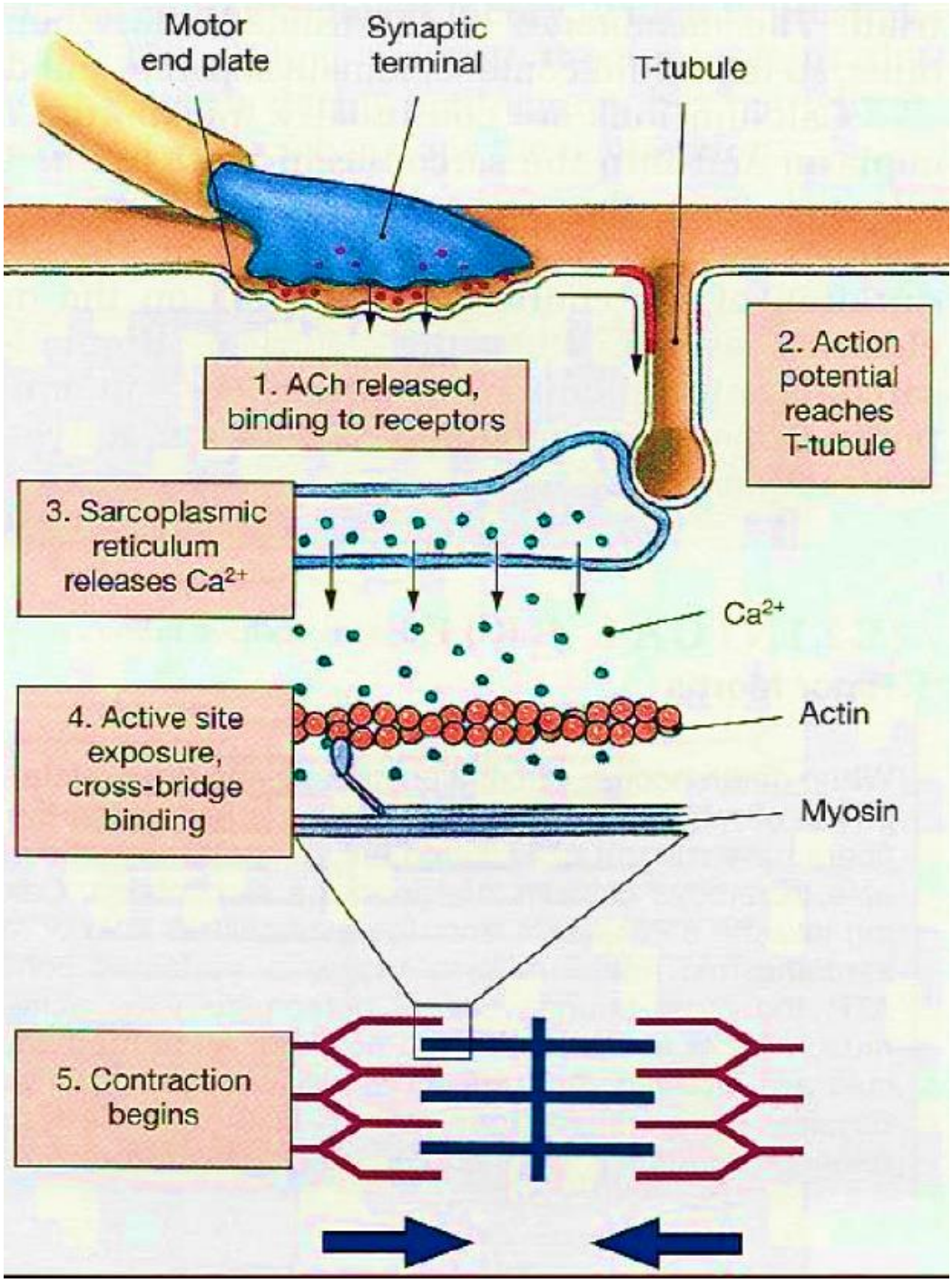
A figura mostra a acetilcolina (ACh) agindo na região pós-sináptica sobre um receptor nicotínico que controla um canal de cátions e também sobre um receptor nicotínico pré-sinpático que atua facilitando a libreação de ACh durante atividade sináptica sustentada. A terminação nervosa também contém acetilcolinesterase, quando essa enzima é inibida há aumento da quantidade de ACh livre e da taxa de extravasamento de ACh por meio do transportador de colina. Em condições normais, esse extravasamento de ACh é insignificante.
ACh, Acetilcolina; AcCoA, acetil-coenzima A; AChE, acetilcolinesterase; CAT, colina acetiltransferase; CoA, coenzima A. Fonte: Rang & Dale's Pharmacology, 6 ed.. ISBN: 0443069115 Ed. Churchill Livingston.

1.3 Contração Muscular

A Contração muscular é um processo fisiológico característico das fibras musculares que corresponde à capacidade de gerar tensão com a ajuda de um neurônio motor. Na contração muscular, a actina desliza sobre os filamentos da miosina, que conservam seus comprimentos originais. A contração se inicia na faixa ansiotrópica, ou A, onde a actina e a miosina se sobrepõem.

O impulso elétrico chega às terminações nas fibras musculares pelo nervo motor através do potencial de ação. Nessas terminações o nervo secreta uma quantidade pequena de substancia que irá conduzir o estímulo elétrico essa substância é a acetilcolina. A acetilcolina irá agir em um local da membrana da fibra muscular para abrir múltiplos canais. A abertura desses canais fará com que ocorra a difusão de grande quantidade de íons sódio para o lado de dentro da membrana das fibras musculares. É essa perfusão que desencadeia o potencial de ação da membrana. O potencial se propaga por toda a extensão da membrana de fibra muscular e é conduzida pelo centro da fibra. Os filamentos de miosina e actina se movimentam e desencadeiam o processo contrátil através de forças atrativas ativadas pelos íons cálcio. Após um alguns segundos, os íons cálcio voltam para o reticulo sarcoplasmático, por um sistema de bomba específico. Lá permanecem armazenados até que novo potencial de ação muscular inicie. É essa retirada de íons cálcio das miofibrilas que faz com que a contração muscular cesse.

Figura 3 – Ilustração do Mecanismo de Contração Muscular



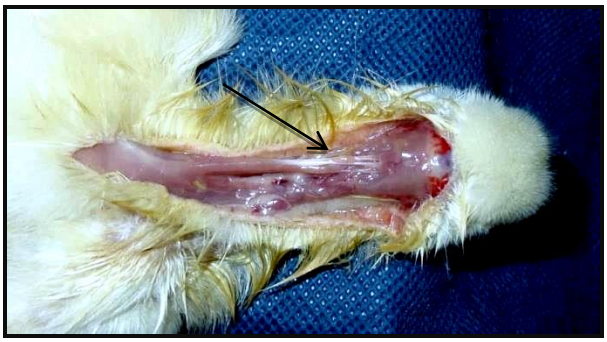
A contração de um músculo resulta do encurtamento de suas fibras, o que por sua vez resulta do encurtamento dos filamentos de actina e miosina, que ativamente deslizam e se encaixa um entre o outro.

Investigações em camundongos transgênicos sugerem que a desnervação do músculo da JNM esta relacionada com o inicio da ELA (DUPUIS & LOEFFER, 2009; DOBROWOLNY, 2005). Também foi mostrado a interferência do Riluzole nos AChRs de camundongo em concentrações muito elevadas (MOHAMMADI *et al.*, 2002), e em concentrações clinicamente utilizadas (0,5 a 5 µM; revisado em BELLINGHAM, 2011). Recentemente, Deflorio e colaboradores (2012) demonstrou o bloqueio dos AChRs em oocitos de Xenopus e células HEK.

A preparação isolada do músculo biventer cervicis (MBC) de pintainhos (Figura 3), é vastamente usada como modelo experimental de JNM. A resposta de contração muscular de aves tem sido usada para estudar as ações de drogas agonistas nicotínicos, assim como músculo de rã (STURKIES, 1999). Podendo ser usada para testar simultaneamente tanto atividade de bloqueio neuromuscular, indicado através da redução da contração produzida pela estimulação dos nervos, como para a atividade de despolarização, como indicado pela contração muscular.

Neste trabalho objetivamos mostrar as ações induzidas pelo Riluzole, sobre a JNM de aves, usando-se de parâmetros eletromiográficos.

Figura 4-Fotografia de preparação músculo biventer cervicis (MBC) de Pintainho



Fonte: do Autor

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos induzidos pelo Riluzole sobre a junção neuromuscular de aves.

2.2 Objetivos Específicos

- Verificar o efeito de diferentes doses de Riluzole sobre registros eletromiográficos em preparação nervo músculo biventer cervicis de pintainho.
- Investigar a atividade da enzima acetilcolinesterase de músculos biventer cervicis.
- Investigar o mecanismo de ação do Riluzole sobre os receptores nicotínicos pós-sináptico.
- Investigar os efeitos do Riluzole quanto à dependência de cálcio.

3 MANUSCRITO

Este manuscrito está disposto na forma na qual deverá ser submetido para o Periódico **Muscle & Nerve** (ISSN: 1097-4598) intitulado “Modulation of vertebrate neuromuscular transmission induced by Riluzole in avian nerve muscle preparations”.

Modulation of vertebrate neuromuscular transmission induced by Riluzole in avian nerve muscle preparations

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Abbreviations:
ALS: Amyotrophic Lateral Sclerosis; CNS: Central Nervous System; AChRs: Acetylcholine receptors; ACh: Acetylcholine; FMC: Force of Muscle Contraction; FDA: Food and Drug Administration; GLU: Glutamate; NMJ: Neuromuscular Junction; DMSO: Dimethyl sulfoxide; KCl: Potassium Chloride; d-Tc: d-Tubocurarine; MBC: muscle of chick biventer cervicis; NEO: Neostigmine; PNS: Peripheral Nervous System

Abstract:
Riluzole is the official drug for the treatment of Amyotrophic Lateral Sclerosis (ALS). It is also known as a neuroprotective agent, which possess anticonvulsant, analgesic and anesthetic properties. ALS is related to the degeneration of upper and lower motor neuron and acetylcholine receptors are associated with motor neuron diseases. Because the anatomical advantage of the neuromuscular junction (NMJ) in terms of it increased size, it has been used as a model to study the mechanisms of drugs and toxins that affect the cholinergic neurotransmission. The aim of this study was to investigate the changes induced by Riluzole, on skeletal NMJ of avian, using direct (80v) and indirect (7v) electrical stimulation. In control preparations, only with Krebs solution, no changes in the force of muscle contraction were observed. However, Riluzole induced a dose and time-dependent response. The dose of 5 μM induced a significant decrease in the contractile response ($p<0.05$). At concentration of 10 μM there was a significant facilitation of muscle contraction. Riluzole (10 μM) also interfered in the muscle direct response to electrical stimulation (80v), reversing the muscle blockade promoted by d-tubocurarine. At biochemical assays it was observed an increase in the activity of acetylcholinesterase in the presence of Riluzole, with or without neostigmine. These results show that Riluzole has different mechanisms of action in the peripheral nervous system and it can contribute to the possible use of this drug in the treatment of neuromuscular disorders.

Keywords: Riluzole, neuromuscular junction, acetylcholine receptors

1 Introduction

Riluzole was developed because it possesses antiglutamatergic properties that might reduce exitotoxicity in Amyotrophic Lateral Sclerosis (ALS). Riluzole slowed disease progression in two randomized controlled trials, reducing mortality albeit modestly (GORDON, 2013). First described as an anticonvulsant agent in animal models (BENAVIDES *et al*, 1985), it is currently the only drug approved by the FDA (Food and Drug Administration - USA) for the treatment of ALS. ALS is related to the degeneration of upper and lower motor neuron disease. The disease begins focally in the central nervous system (CNS) and then continuously spread (GORDON, 2013), resulting in progressive muscle wasting and loss of motor function, leading to paralysis and death of affected individuals (JULIEN, 2001).

Riluzole blocks the presynaptic release of glutamate (GLU) at central synapses (MARTIN *et al*., 1993). This mechanism has been proposed as responsible for the neuroprotective drug activity. Since Riluzole has gained the attention of the international scientific community, several mechanisms of action have been described, including inhibition of Na^+ (BELTRAN-PARRAZAL & CHARLES, 2003) channels, activation of K^+ channels (GRUNNET *et al*., 2001) and inhibition of neuronal activity (BELLINGHAM, 2013).

According to Nakanishi (2005), acetylcholine receptors (AChRs) are associated with motoneuron diseases. The blockage of AChRs induced changes in the excitability of motoneurons which are quantitatively similar to axotomy in amplitude and in timing (BICHLER *et al*., 2007). AChRs are the most studied functional proteins at neuromuscular junction (NMJ). Recently it was shown that a defective β-4 subunit of the AChRs would be implicated in the ALS (MORICONI *et al.*, 2011). In this context, the NMJ is a chemical synapse which differs from the central excitatory synapses just because the release of the neurotransmitter acetylcholine (ACh) activates nicotinic receptors which localize at the sarcolemma of skeletal muscles (ARROWSMITH, 2007). Because the anatomical advantage of the NMJ in terms of it increased size, it has been used as a model to study the mechanisms of drugs and toxins that affect the cholinergic neurotransmission (DAL BELO *et al.*, 2002, DAL BELO *et al.*, 2005). GIFONDORWA (2007) demonstrated that the chronic administration of Riluzole was ineffective to improve the locomotor function of transgenic mice of ALS in contrast to previous report of GURNEY (1996). In addition, the former showed an improvement of mice NMJ associated with treatments that counteract the

symptoms of ALS. Hence, the aim of this work was to investigate the mechanisms of Riluzole-induced activity at the vertebrate skeletal neuromuscular junctions.

As far as of our knowledge this is the first work aimed to investigate the mechanisms of Riluzole directly on electrically evoked cholinergic neurotransmission. The results presented herein may improve the knowledge about Riluzole at cholinergic neurotransmission, whilst uncover the potential of this agent as novel candidate to treat neuromuscular disorders.

2 Experimental Section

2.1 Reagents: All reagents (Ach, Riluzole, Neostigmine, d-Tubocurarine and KCl) used are of high purity and were obtained from Sigma Aldrich.

2.2 Animals: male chicks, HY-LINE aged 4-10 days, provided by a commercial source (Agro Sinuelo, São Gabriel, RS, Brazil) and kept in the animal house of the University Federal do Pampa (UNIPAMPA). Animals were maintained in standard animal housing conditions, on a 12-h light/dark cycle, with food and water *ad libitum*. All assays were performed according to the Ethics Committee for the Use of Animais-CEUA/UNIPAMPA, under No. 011/2012 approved protocol.

2.3 Biological and Eletromyographic Assays

2.3.1 Chick biventer cervicis preparation: After euthanasia with halothane, the chick was affixed on a dissection plate in a prone position, the muscles were dissected (GINSBORG & WARRINER, 1960) and mounted in a tub in the isolated organ bath (AVS Instruments, São Carlos/SP, Brazil). Chick biventer cervicis nerve-muscle preparations were mounted (resting tension: 1 g) in basic Krebs solution at 37°C containing (in mM): 120 NaCl, 2 KCl, 2,5 $CaCl_2$, 1.20 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose and 2.6 $NaHCO_3$-pH 7.4, aerated constantly with a carbogênico mixture (95% O_2 and 5% CO_2) and allowed to stabilize for 30 min prior to use. Supramaximal electric stimuli indirect (0.1Hz, 0.2ms, 7v) or direct (0.1Hz, 2ms, 80v) were applied through bipolar platinum electrodes connected to an electrical stimulator (AVS Instruments, São Carlos, SP). Recordings of the amplitude of muscle contraction force (FMC) were taken during 120 min and evoked responses to ACh (110 µM) and KCl (20 mM) (FERREIRA, *et al.*, 2011) before and after Riluzole incubation.

2.3.2 Riluzole treatment: Riluzole was first diluted in DMSO and further dilutions made in KREBS solution. The Riluzole doses used were (in µM): 5, 10 and 20, in 0.1% DMSO final concentration. All treatments were done with 7v (stimuli indirect) unless otherwise noted.

2.3.3 Low calcium assay: After stabilization of the recordings, the basic Krebs medium was replaced by Krebs with 0.5 mM calcium, following the curves of ACh and KCl were made and recording taken with or no Riluzole at 10 µM.

2.3.4 Neostigmine assay: The muscle was treated with Riluzole (5 and 10 µM) for 30 min and then neostigmine 0.5 µM was added and recordings followed for 120 min.

2.3.5 d-Tubocurarine (d-Tc) assay: *Direct stimulation:* it was added d-Tc 20 µM for a total blockade of muscle contraction, the voltage was then exchanged from 7v to 80v and 10 µM Riluzole was added up. *Indirect Stimulation:* d-Tc 10 µM was added at 7v to the partial block (50%), and then added Riluzole 10 µM and recordings followed for 120 min.

2.3.6 Acetylcholinesterase (AChE) assay: After the eletromiographic recordings the muscles were homogenized and assayed for cholinesterase activity according to ELLMAN and co-workers (1961).

2.4 Statistical Analysis

Results were expressed as mean ± standard error. The significance of differences was determined by ANOVA test, with $p<0.05$ considered significant.

3 Results and Discussion

The *in vitro* preparation of chick muscle biventer cervicis (MBC) is widely used to prove the effects of drugs and toxins (STÄNDKER *et al.*, 2012) that affect pre-and postsynaptic locations NMJ (GINSBORG 1960; HARVEY *et al.*, 1994). Due to the high density of acetylcholine receptors (AChRs) in the chick muscle, the response to ACh allows the evaluation of the affinity of chemical compounds at these receptors that are localized at the sarcolemmal region of the endplate (BRAGA *et al.*, 2013). This work shows the biochemical and pharmacological activities induced by Riluzole at avian neuromuscular

preparations. The doses of 5, 10 and 20 μM were assayed and induced paradoxal inhibitory and facilitatory effects respectively, at muscle twitch-tension (Figure 1a). When the concentration of 5 μM (n=6) was assayed there was a maximum blockage of 10 ± 1% of the muscle twitches at 120 min recordings. However, for the concentration of 10 μM (n=6) there was a significative increase of the muscle twitches in 120 min recordings ($p<0.05$). In contrast, no alteration of the muscle strength was observed when the maximum concentration of Riluzole (20 μM, n=6) was tested ($p<0.05$) compared with the control-Krebs. The curves of the exogenous applied ACh (110 μM) showed a increase induced by the Riluzole (Figure 1b). In the concentration of 5 μM Riluzole there was an increase of 5 ± 1% compared to the control Krebs ($p<0.05$), however at 10 μM, there was a further increase in the amplitude of contracture to (20 ± 2%, $p<0.05$), that was increased to 25 ± 2% at Riluzole (20 μM, $p<0.05$).

The data related to the direct effect of Riluzole at NMJ show both inhibitory to excitatory actions. Indeed, there are in the literature examples of drugs that can play disrupting and improving the neuromuscular transmission (SU *et al.,* 2011). The inhibitory actions of Riluzole at MBC resemble drugs that affect ionic channels, as do many animal venoms (RASH *et al.,* 2000; WICKRAMARATNA, *et al.,* 2003). Indeed, it is thought that Riluzole inhibits Na^{+} and Ca^{++} channels at motoneurons of rats CNS (LAMANAUSKAS & NISTRI, 2008). Recently, Palma and co-workers, (2011) used the technique of expression of of muscle AChRs in Xenopus oocytes to study their function in ALS patients under treatment with Riluzole. They have shown that Riluzole affects the AChRs by prolonging their closing time. Palma and co-workers (2011) also showed that Riluzole affects the single channel ACh–evoked responses using cells expressed with AChRs. Therefore, in our experimental conditions, Riluzole also promoted an increase of muscle twitch tension, with a significant increase in the ACh-evoked contractures. Our data, mostly by the increase of muscle contractures to the ACh, clearly corroborates the literature showing that Riluzole may affect the endplate response by interact directly with the muscle AChRs.

At NMJ, calcium is the trigger of several signaling events, which ultimately lead to the fusion of neurotransmitter vesicles to the presynaptic membranes and the release of ACh and other molecules to synaptic cleft (ARROWSMITH, 2007). In this regard Riluzole is thought to directly blocks with P/Q type Ca^{2+} channels but not the L-type (CAO *et al.,* 2002). Figure 2 shows the dependence of Ca^{2+} for the Riluzole interference at NMJ. When the muscle was treated with low-calcium Krebs solution (0.5 mM - BRAGA *et al.,* 1991), there was an inhibition of the muscle twitch increase, induced by (10 μM) Riluzole. This effect first suggests that the positive interaction of Riluzole with muscle endplates is calcium dependent.

Second, even calcium channels may be blocked by Riluzole at endplate, the increase of muscle twitch-tension and consequent decrease in low-calcium preparations reveal that there will be a more strong and specific site for Riluzole interation at endplate.

In addition, experiments carried out in the presence d-Tubocurarine at low concentrations (d–Tc 10 µM), which partially blocks the amplitude of the muscular twitches (WEBB & BOWMAN, 1974), and at high concentrations (d-Tc 20 µM), revealed that Riluzole was able to reverse the neuromuscular blockade (Figure 3). This later activity is strikingly to understand the mechanisms involved in the facilitatory action of Riluzole at NMJ. Several drugs are listed in literature in terms of anti-curaremimetic effect. For example, physostigmine and neostigmine (NEO) are indirect modulators of ACh release, while carbamilcholine acts direct at the AChRs (ARROWSMITH, 2007). Another example is those drugs that directly affect the amount of ACh release. To date, prednisolone, a corticosteroid that directly affect the neuromuscular transmission of rats, reverses the d-Tc-induced blocked of muscle twitches by probably increases the amount of ACh released at endplates (DAL BELO *et al.*, 2002).

Riluzole also induced contradictory modulations of the AChE enzyme activity. AChE is a regulatory enzyme responsible for completing the transmission of nerve impulses at synapses via the hydrolysis of the neurotransmitter ACh. Because of this, AChE has a key role in regulating the transmission of nerve impulses and, if inhibited, usually induces the death of the organism by a cholinergic intoxication (ALBUQUERQUE *et al.*, 2006). NEO is an indirect cholinergic modulator that inhibits reversibly the AChE activity (WOO, 2013). In our experimental condition, NEO (0.5 µM) reversed the blockage of muscle twitches (10±2%) induced by Riluzole 5 µM ($n=6$, $p<0.05$) (Figure 4). The increase in the contraction of Riluzole 10 µM was potentiated in almost 30% in the presence of NEO. This increase observed in figure 4 at 5 µM and 10 µM showed a contradictory increase in the AChE activity (Figure 5). This result reveals antagonistic actions of Riluzole with nerve-terminal AChE. The interaction with NEO, also suggests that Riluzole may interact in another site other than the esterasic site of the AChE enzyme (HÖRNBERG *et al.*, 2007).

4 Conclusion

In conclusion, Riluzole affected the vertebrate neuromuscular by different manners: in low concentrations induced a decrease of muscle twitches, with an increase of AChE activity. In higher concentrations, induced facilitatory actions, which may be related to a positive

modulation of the neurotransmitter release or accumulation of ACh at the endplate. Despite the contradictory actions of Riluzole at NMJ, the present work revealed the potential of this drug to treat neuronal disorders related to the peripheral nervous system (PNS), with care in the administration of precise concentrations.

5 Acknowledgements

The authors thank the Coordenação de Aperfeiçoamento de Pessoal (CAPES) for AP Lucho fellowship and by financial support by means of Edital 063/2010 Toxinologia. Thank the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), by financial support through Edital PRONEM/FAPERGS/CNPq 063/2010 and thank the Universidade Federal do Pampa (UNIPAMPA) for fellowship to AP Perin, through the Programa de Bolsas de Desenvolvimento Acadêmico (PBDA). The authors also acknowledge MSc. Ana Paula Zemolin for assistance in the biochemical experiments.

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FIGURES:

Figure 1.

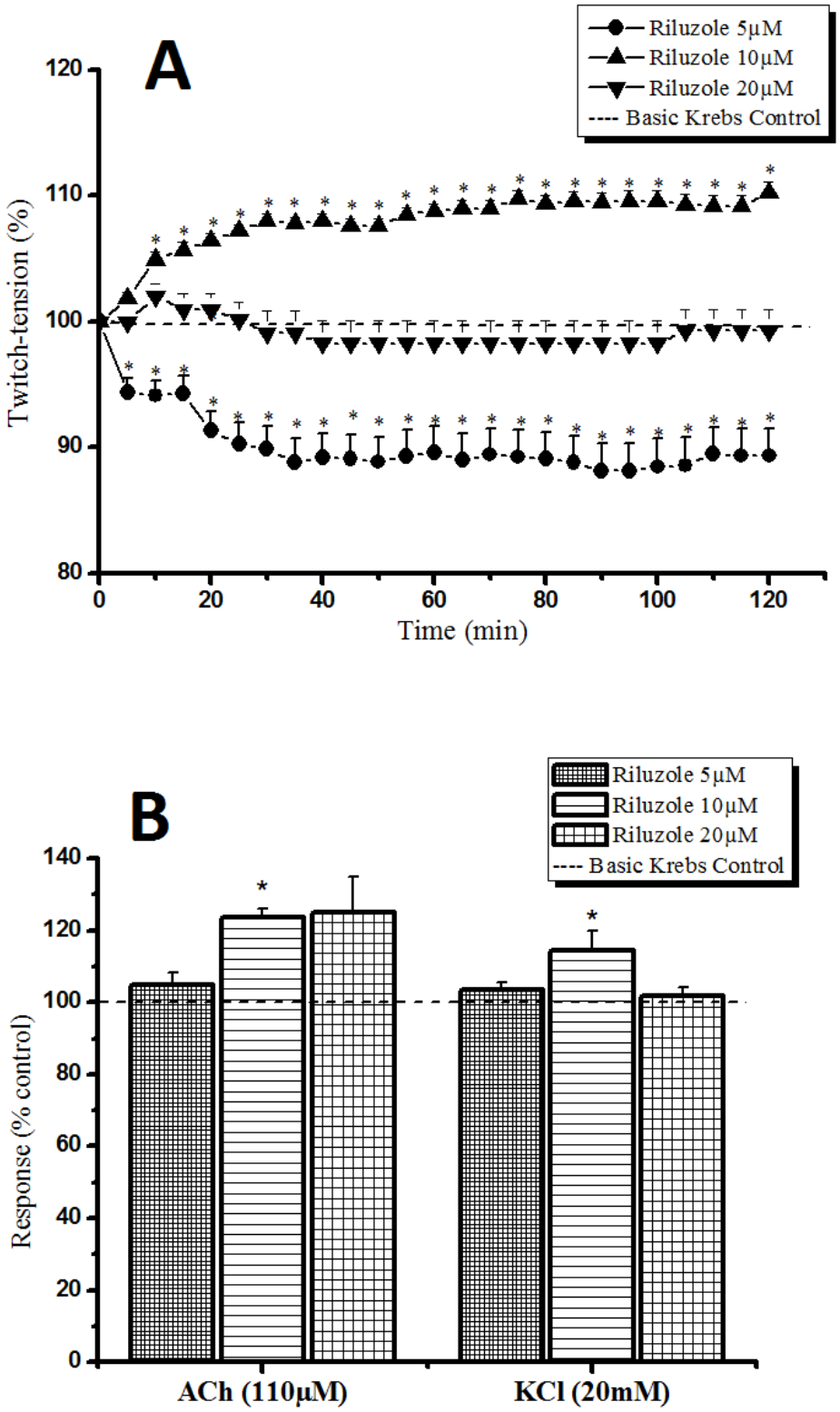


Figure 2.

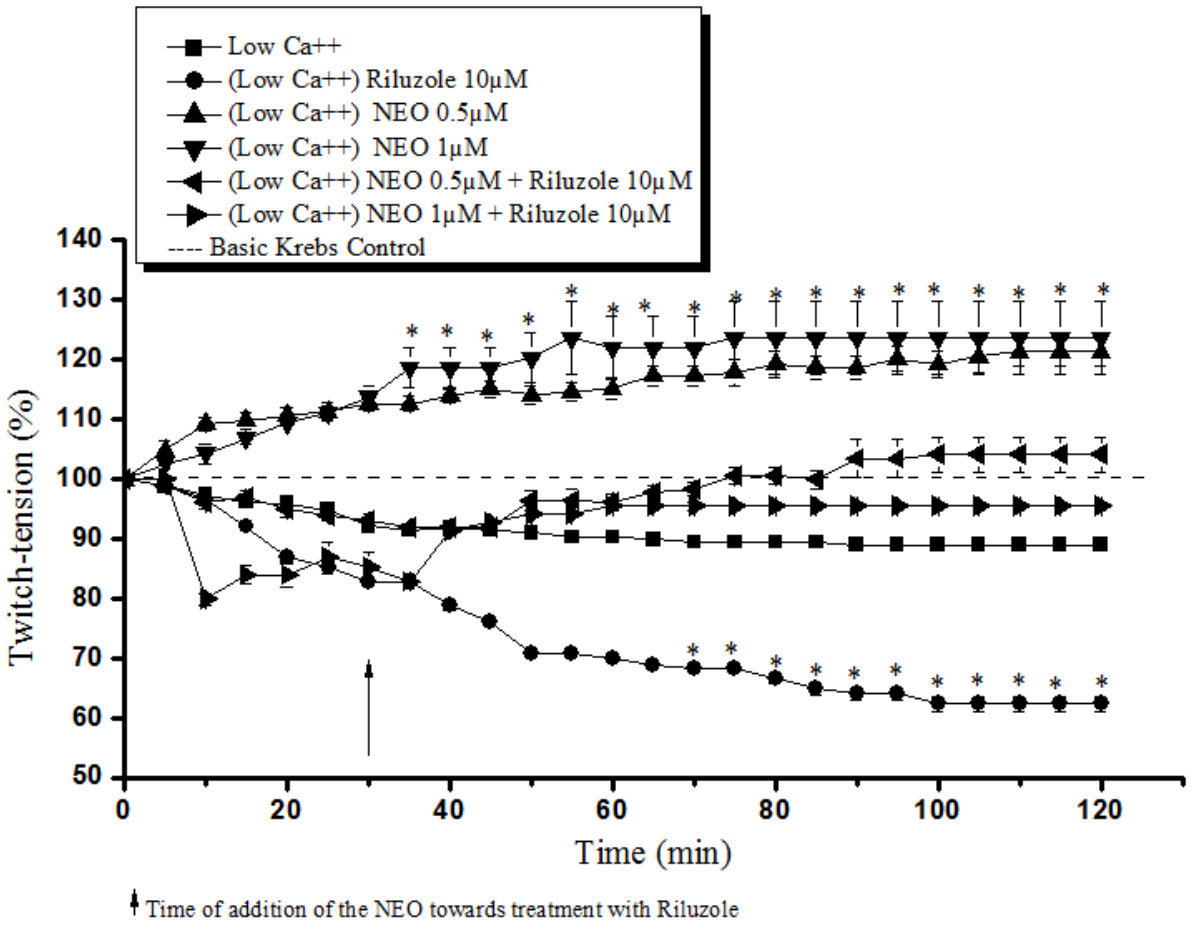
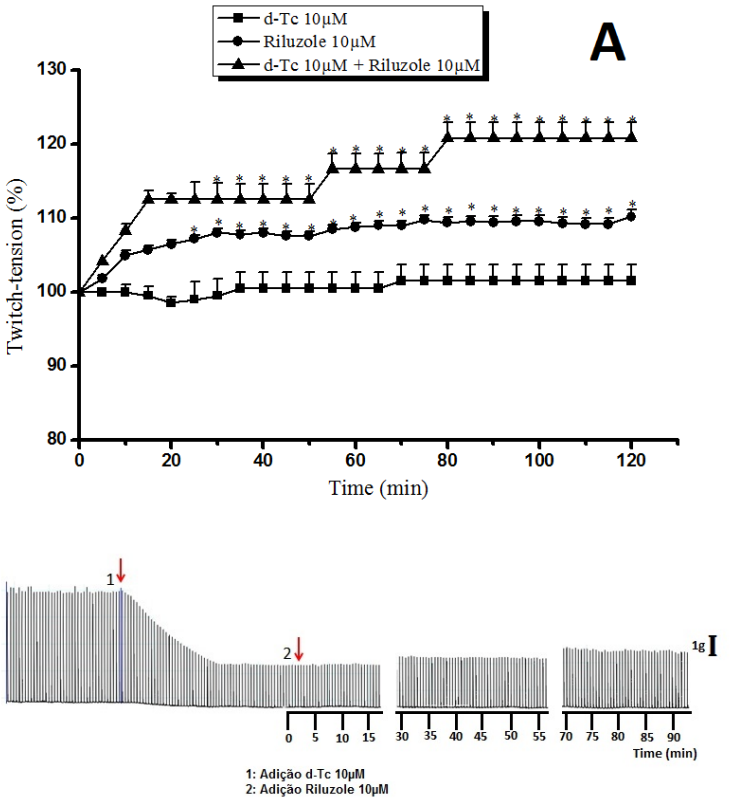

Low Ca++
(Low Ca++) Riluzole 10µM
(Low Ca++) NEO 0.5µM
(Low Ca++) NEO 1µM
(Low Ca++) NEO 0.5µM + Riluzole 10µM
(Low Ca++) NEO 1µM + Riluzole 10µM
---- Basic Krebs Control
Twitch-tension (%)
Time (min)
Time of addition of the NEO towards treatment with Riluzole

Figure 3.


d-Tc 10µM
Riluzole 10µM
d-Tc 10µM + Riluzole 10µM
A
Twitch-tension (%)
Time (min)
1
2
1g
Time (min)
1: Adição d-Tc 10µM
2: Adição Riluzole 10µM

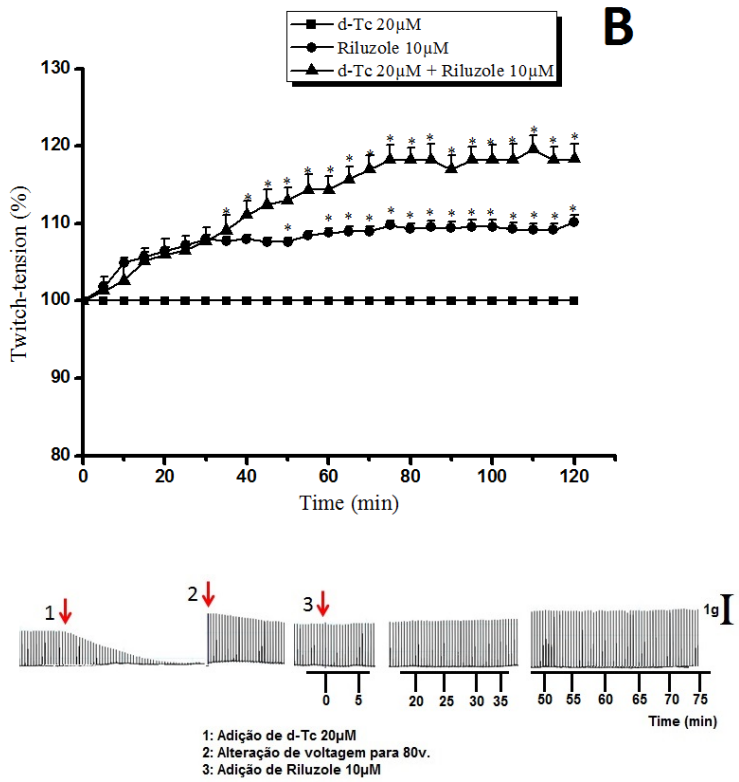

d-Tc 20µM
Riluzole 10µM
d-Tc 20µM + Riluzole 10µM
B
Twitch-tension (%)
Time (min)
1
2
3
1g
Time (min)
1: Adição de d-Tc 20µM
2: Alteração de voltagem para 80v.
3: Adição de Riluzole 10µM

Figure 4.

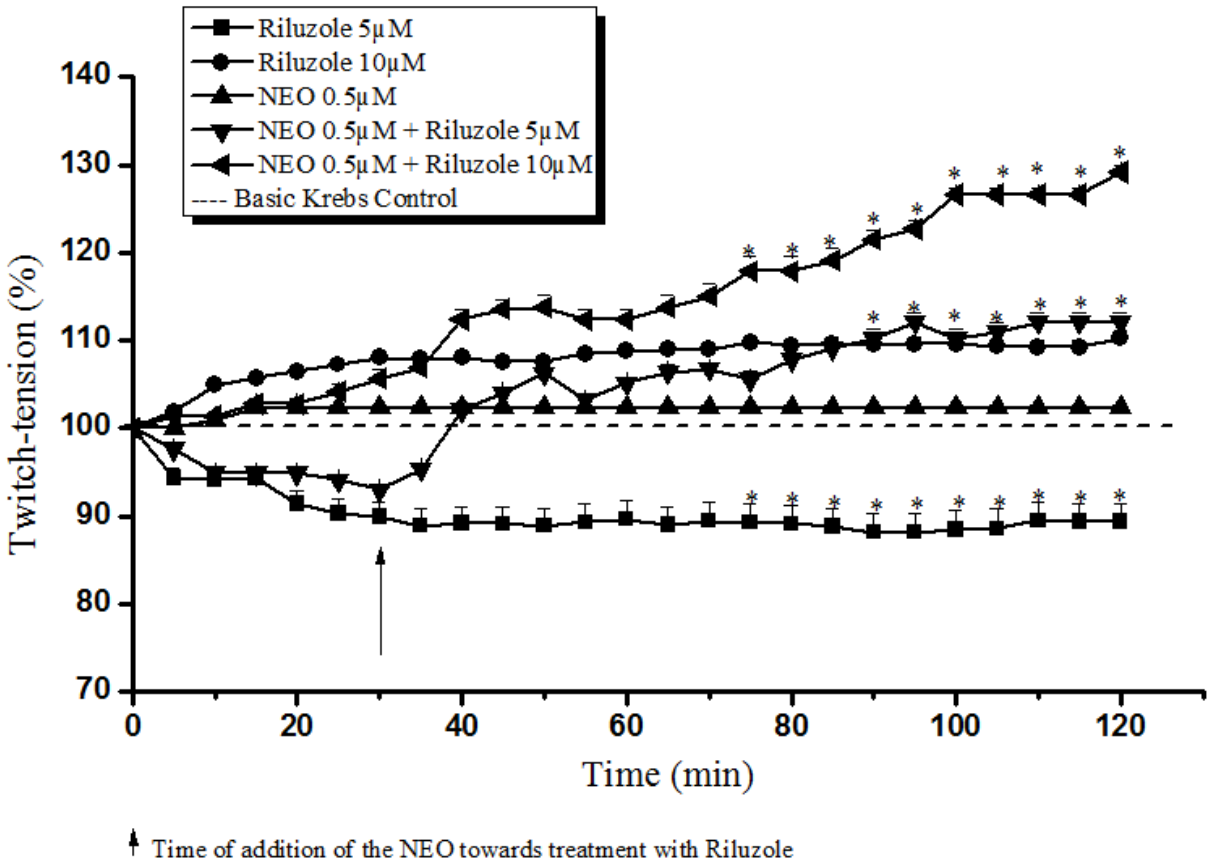

Riluzole 5µM
Riluzole 10µM
NEO 0.5µM
NEO 0.5µM + Riluzole 5µM
NEO 0.5µM + Riluzole 10µM
---- Basic Krebs Control
Twitch-tension (%)
Time (min)
140
130
120
110
100
90
80
70
0
20
40
60
80
100
120
Time of addition of the NEO towards treatment with Riluzole

Figure 5.

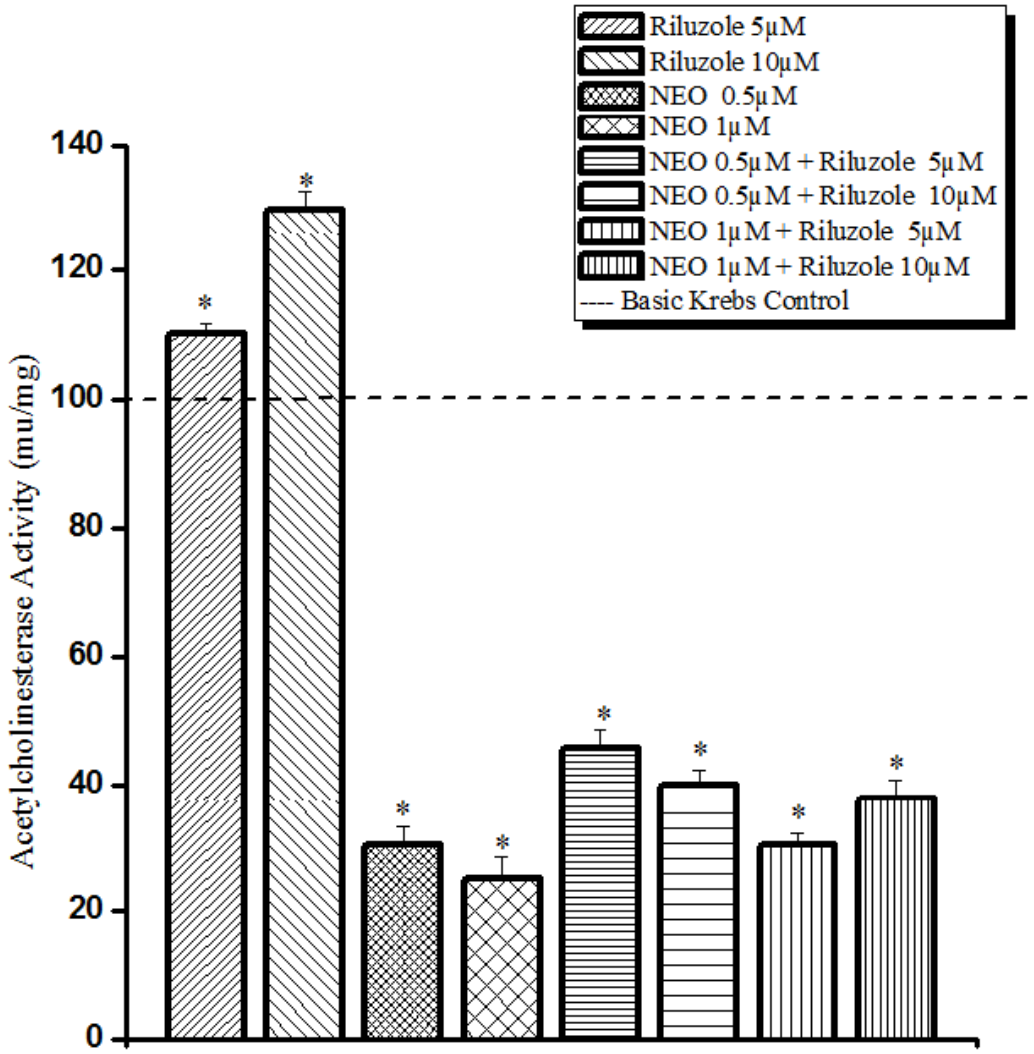

Riluzole 5µM
Riluzole 10µM
NEO 0.5µM
NEO 1µM
NEO 0.5µM + Riluzole 5µM
NEO 0.5µM + Riluzole 10µM
NEO 1µM + Riluzole 5µM
NEO 1µM + Riluzole 10µM
---- Basic Krebs Control
Acetylcholinesterase Activity (mu/mg)
140
120
100
80
60
40
20
0
*

Figure 1. Representative graphs of the effect of Riluzole (5, 10 and 20 µM) on chick muscle biventer cervicis preparation with indirect electrical stimulation in comparison with basic Krebs medium (control 100%) (mean ± standard error of 6 experiments). **A)** Contractile response of Riluzole treatments, where it is noticed at 10 µM a facilitation and at 5 µM a decreased contraction. **B)** Curves of ACh (110 µM) and KCl (20 mM) after Riluzole treatment. *Significance at $p<0.05$.

Figure 2. Representative graph of the effect of low calcium treatment with Riluzole in preparation chick biventer cervicis under indirect electrical stimulation (basic Krebs medium = control 100%) (mean ± standard error of 6 experiments). The muscles were maintained with low calcium (0.5 mM) and treated with Riluzole (10 µM) and Neostigmine (NEO 0.5 and 1 µM).The treatment with Riluzole (10 µM) in low calcium medium showed a significant decrease in the muscle contraction. Neostigmine (0.5 or 1 µM) with or without Riluzole showed a raise in the muscle contraction in relation to low calcium condition. *Significance at $p<0.05$ when compared to Krebs with low calcium.

Figure 3. Representative graph of the effect of d-Tubocurarine in chick muscle biventer cervicis in the presence of Riluzole under direct or indirect stimulation (basic Krebs medium = control 100%) (mean ± standard error of 6 experiments). Below the graphs it is shown representative image of electromyographic records **A)** Contractile response from treatment with Riluzole (10 µM) and addition of d-Tubocurarine (10 µM) under indirect stimulation (1ms, 0.1 Hz, 7v). **B)** Contractile response from treatment with Riluzole (10 µM) and addition of d-Tubocurarine (20 µM) under direct stimulation (2ms, 0.1 Hz, 80v). *Significance at $p<0.05$ when compared to the d-Tubocurarine.

Figure 4. Representative graph of the effect of Riluzole treatments with neostigmine (NEO) in chick muscle biventer cervicis with indirect electrical stimulation, in comparison with those obtained only with Krebs (control 100%) (mean ± standard error of 6 experiments). Effect of NEO 0.5 µM in the presence of Riluzole (5 and 10 µM), showing an increase in the contractile response. The arrow shows the moment of NEO addition. *Significance at $p<0.05$.

Figure 5. Representative graph of acetylcholinesterase (AChE) activity in chick biventer cervicis muscle after Riluzole (5 and 10 µM) treatments and neostigmine (NEO - 0.5 µM), with indirect stimulation, in comparison with those obtained only with Krebs (control 100%) (mean ± standard error of 6 experiments). *Significance at $p<0.05$.

4 CONSIDERAÇÕES FINAIS

Com base nos dados obtidos neste trabalho, podemos considerar:

- Aumento na contração muscular com a dose de 10 µM, com estimulo indireto.
- Bloqueio na contração muscular com a dose de 5 µM, com estimulo indireto.
- Interferência do Riluzole com os receptores de acetilcolina (AChRs).
- Aumento da resposta a ACh exógena, nas curvas pós tratamento com Riluzole, em ordem crescente: 5, 10 e 20 µM.
- Efeito da droga pré e pós sinapticamente, através de estimulo direto e indireto.
- Riluzole é depentente de cálcio.
- A ação do bloqueador da enzima AChE, NEO 0,5 µM, em JNM de pintainho.
- A ativação da enzima AChE quando tratada com Riluzole nas doses de 5 e 10 µM e NEO 0,5 µM.

5 PERSPECTIVAS

Nossas perspectivas futuras, com base nos resultados que obtemos, são:

- Testar outros inibidores de AChE como tacrina e organofosforados.
- Testar a Pralidoxima (reversor da inibição de AChE).
- Investigar a expressão do peptídeo relacionado ao gene da calcitonina (CGRP) através de imunodetecção, após o tratamento com o Riluzole. Sendo este um peptídeo localizado no compartimento da região pré-sináptica das junções neuromusculares dos músculos esqueléticos. (MATEOLLI *et al.,* 1990).
- Concluir os testes de AChE em homogenatos de cabeça de barata frente ao Riluzole.

CAPÍTULO II

Efeito do Riluzole no Sistema Nervoso Central em Preparação de Fatias Hipocampais de Camundongo

6 INTRODUÇÃO

6.1 Serpente do Gênero Crotalus

As serpentes do gênero Crotalus, popularmente conhecidas por cascavel, boicininga ou maracabóia, são responsáveis por 10 a 20% dos acidentes ofídicos em todo país, chegando a quase 30% em regiões como Botucatu (OLIVEIRA, 2007). Essas serpentes normalmente são encontradas em campos abertos, áreas secas, arenosas e pedregosas e são raras nas faixas litorâneas (PINHO *et al*., 2000). São facilmente identificadas por possuírem na porção terminal da cauda um guizo ou chocalho (Figura 4), característica peculiar da espécie, (PINHO *et al.*, 2000) cabeça triangular, olhos pequenos com pupila em fenda, presença de fosseta loreal e escamas na cabeça, dentição solenóglifa (Figura 5), cauda com guizo ou chocalho (Centro Virtual de Toxinologia, www.cevap.org.br).

O gênero Crotalus caracteriza-se por pertencer à família Viperidae, a qual incluem também os gêneros Bothrops, Lachesis, Porthidium e Bothriopsis, que possuem o aparelho peçonhífero mais complexo e classificam-se como solenóglifas por possuírem presas móveis na região frontal da boca.

Figura 5- Fotografia de Serpente da Espécie *Crotaluss durissus terrificus*

Fonte: http://www.reptile-database.org

Figura 6- Fotografia de Dentição das Serpentes da Família Viperidae.

Fonte: http://www.reptile-database.org

6.2 Venenos

A subespécie *Crotalus durissus terrificus* (Laurenti, 1768), cascavel brasileira, é a segunda mais importante causa de acidentes ofídicos no Brasil (NOGUEIRA *et al.* 2007), tendo seu veneno investigado desde 1938 (SLOTTA & FRAENKEL-CONRAT, 1938). A composição do veneno é uma mistura de enzimas, toxinas (crotamina crotoxina, giroxina, convulxina) e peptídos (BARRAVIEIRA *et al.*, 1995) que induzem neurotoxicidade, distúrbios de coagulação, miotoxicidade sistêmica e insuficiência renal aguda.

A toxicidade decorre principalmente devido a presença de fosfolipase A_2 (crotoxina) (SITPRIJA & CHAIYABUTR, 1999). As fosfolipases A_2 (PLA 2) são um complexo heterodimérico neurotóxico, que produzem paralisia progressiva e mionecrose em concentrações elevadas (BREITHAUPT, 1976; MONTECUCCO *et al.*, 2008). A crotamina é a segunda principal toxina no veneno de Cdt, uma base de baixo peso molecular desprovido da atividade de PLA 2 (OWNBY, 1998), com uma ação específica sobre os canais de sódio sensíveis à voltagem de células de músculos (VITAL-BRASIL & FONTANA, 1993).

Os efeitos sistêmicos do veneno de *Crotalus durissus terrificus (Cdt)* residem principalmente na neurotoxicidade periférica, porém, quando injetado diretamente sobre o SNC de mamíferos, induz convulsões e morte (MELLO & CAVALHEIRO, 1989). Nos terminais nervosos, crotoxina, principal componente do veneno, induz alterações na liberação do transmissor a uma diminuição lenta e progressiva resultando em um bloqueio neuromuscular total (CHANG & LEE, 1977;. SIMIONI *et al.*, 1990).

Assim, a busca de novos inibidores de veneno, naturais ou sintéticos que atuem sobre o sistema nervoso central e periférico e que seja capaz de complementar a soroterapia atual de modo a neutralizar os danos restantes de envenenamento de cobra, se faz necessária.

6.3 Neuroproteção

Neuroproteção refere-se a estratégias utilizadas para preservação da estrututa ou função neuronal contra lesões ou degeneração do SNC e SNP, após distúrbios agudos (por exemplo, acidente vascular cerebral ou lesão do sistema/trauma nervoso) ou como resultado de doenças neurodegenerativas crônicas (Mal de Parkinson, Alzheimer, Doenças do Neurônio Motor).

Apesar das diferenças nos sintomas ou lesões associadas com distúrbios do sistema nervoso, muitos dos mecanismos da neurodegeneração são os mesmos podendo ser utilizados em mais de uma desordem. Mecanismos comuns incluem o aumento dos níveis de estresse oxidativo, disfunção mitocondriais, excitotoxicidade, alterações inflamatórias, acumulação de ferro e agregação de proteínas.

Os mecanismos exatos da ação dos extratos de plantas permanecem em grande parte desconhecidos, no entanto, um número de relatórios anteriores indica que os compostos derivados de plantas, tais como ácido rosmarínico, a quercetina e glicirrizina podem inibir as atividades biológicas dos alguns venenos de cobra *in vivo* e *in vitro*. Extratos de *Hypericum brasiliense* (HBE) possuem atividades anti-inflamatórias (PERAZZO *et al.*, 2008a), antibacteriano (ROCHA *et al.*, 1995; FRANCA *et al.*, 2009), antidepressiva (PERAZZO *et al.*, 2008b), e proteção contra a letalidade de *Bothrops jararaca* em camundongos (ROCHA *et al.*, 1991). Os flavonóides derivados de plantas ou extratos de chá afetam a liberação de acetilcolina, contração muscular ou atividade da junção neuromuscular. Especialmente, a quercetina inibe correntes de placa terminal na junção neuromuscular de rato (LEE *et al.*, 2011).

A ação da quercetina no SNC tem sido relatada em diferentes tipos de insultos. Tem sido demonstrado exibir algumas propriedades farmacológicas, tais como anti-inflamatório de redução, na insuficiência cardíaca e propriedades anticancerígenas. Outros estudos, também demonstraram a ação protetora da quercetina na isquemia/reperfusão hepática e renal com os mesmos efeitos antioxidantes (SINGH *et al.*, 2004; POLAT *et al.*, 2006).

7 OBJETIVOS

7.1 Objetivo Geral

Avaliar os efeitos neuroprotetores de *Hypericum brasiliense* (HBE) e seu principal componente, quercetina, em insulto causado por veneno de serpente *Crotalus durissus terrificus* (Cdt).

7.2 Objetivos Específicos

- Investigar o efeito protetor do HBE em fatias de hipocampo e diafragma de camundongo contra veneno de Cdt.
- Investigar o efeito da quercetina em fatias de hipocampo contra o veneno de Cdt;
- Investigar o efeito da quercetina em músculo diafragma sobre injuria de crotamina e crotoxina.

8 MANUSCRITO

Este manuscrito está disposto na forma na qual foi publicado no Periódico BioMed Research International, Volume 2013, Article ID 943520, 6 intitulado como "*In Vitro* Antiophidian Mechanisms of *Hypericum brasiliense* Choisy Standardized Extract: Quercetin-Dependent Neuroprotection".

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 943520, 6 pages
http://dx.doi.org/10.1155/2013/943520

Research Article

In Vitro Antiophidian Mechanisms of *Hypericum brasiliense* Choisy Standardized Extract: Quercetin-Dependent Neuroprotection

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Received 20 September 2013; Accepted 4 December 2013

Academic Editor: Marina Soković

The neuroprotection induced by *Hypericum brasiliense* Choisy extract (HBE) and its main active polyphenol compound quercetin, against *Crotalus durissus terrificus* (Cdt) venom and crotoxin and crotamine, was enquired at both central and peripheral mammal nervous system. Cdt venom (10 μg/mL) or crotoxin (1 μg/mL) incubated at mouse phrenic nerve-diaphragm preparation (PND) induced an irreversible and complete neuromuscular blockade, respectively. Crotamine (1 μg/mL) only induced an increase of muscle strength at PND preparations. At mouse brain slices, Cdt venom (1, 5, and 10 μg/mL) decreased cell viability. HBE (100 μg/mL) inhibited significantly the facilitatory action of crotamine (1 μg/mL) and was partially active against the neuromuscular blockade of crotoxin (1 μg/mL) (data not shown). Quercetin (10 μg/mL) mimicked the neuromuscular protection of HBE (100 μg/mL), by inhibiting almost completely the neurotoxic effect induced by crotoxin (1 μg/mL) and crotamine (1 μg/mL). HBE (100 μg/mL) and quercetin (10 μg/mL) also increased cell viability in mice brain slices. Quercetin (10 μg/mL) was more effective than HBE (100 μg/mL) in counteracting the cell lysis induced by Cdt venom (1 and 10 μg/mL, resp.). These results and a further phytochemical and toxicological investigations could open new perspectives towards therapeutic use of *Hypericum brasiliense* standardized extract and quercetin, especially to counteract the neurotoxic effect induced by snake neurotoxic venoms.

1. Introduction

An estimated 5.4-5.5 million people are bitten by snakes each year, resulting in about 400.000 amputations and about 125.000 deaths [1, 2]. The problem of human suffering by snake bite is actually so relevant that WHO has included it in the list of neglected tropical diseases in April, 2009 [3].

Snake venoms embody a complex mixture of toxic enzymes and proteins, such as myotoxins, neurotoxins, cytotoxins, hemorrhagic metalloproteases, clotting serineproteases, and others [4]. Among all snake venoms, the crotalic is one of the most neurotoxic, in which systemic effects reside primarily in the peripheral neurotoxicity. However, when injected directly on CNS of mammals it can induce convulsion and death [5]. Among other symptoms, the neurotoxicity induced by *Crotalus* poisoning in both central and peripheral nervous system is mainly related to the presence in the venom of the toxins crotoxin [6] and crotamine [7]. Thus, the search of novel venom inhibitors is therefore relevant, being natural or synthetic, in order to complement the current serum therapy and to neutralize the remaining damages of snake envenomation.

Hypericum brasiliense is an annual cycle plant, recurrent in the southern and southeastern Brazil, known by the common names of "milfurada", "milfacadas," and "alecrim bravo" [8, 9]. *H. brasiliense* extract has shown anti-inflammatory and analgesic [10] activities, with contradictory signs on the CNS [11] and protection of mice against lethality of *Bothrops jararaca* venom [12].

The present work demonstrates the ability of *Hypericum brasiliense* standardized extract and quercetin to counteract neurodegenerative insults induced by Cdt venom in brain and muscles preparations. In addition, it is shown that the major neurotoxic components of the *Crotalus durissus terrificus* venom, crotoxin and crotamine, also had their effects prevented in the neuromuscular paralysis at mouse nerve-muscle preparations.

2. Experimental

2.1. Reagents and Venom. All chemicals and reagents used were of the highest purity and were obtained from Sigma, Aldrich, Merck or BioRad. *Crotalus durissus terrificus* venom, crotamine and crotoxin were donated by Dr. S. Marangoni (UNICAMP) and quercetin by Dr. L. Rocha (UFF).

2.2. Animals. Adult Swiss white mice (28–35 g) from both sexes were supplied by the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and by the animal facility from Universidade Federal de Santa Maria (UFSM). The animals were housed at 25°C with access *ad libitum* to food and water. These studies have been done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.3. Plant Material. Hypericum brasiliense leaves were collected in the city of Nova Friburgo, RJ, Brazil, in 2001. A voucher specimen (n°19980) has been deposited at the herbarium of the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil.

2.4. Chemical Analysis. The preparation of *H. brasiliense* EtOH extract (HBE) and detection of its chemical composition were carried out as described elsewhere [13]. Briefly, the chemical analysis was performed with a Liquid Chromatograph (GBC Scientific Equipment LLC, Hampshire, IL, USA), equipped with a Nucleosil MN 120-5 C_{18} silica column (Macherey-Nagel Inc., Bethelehem, PA, USA). The elution was made at room temperature using a linear gradient from 10–60% of acetonitrile in trifluoroacetic acid (0.05% v/v) at a flow rate of 1.0 mL/min in 30 minutes. Peaks were monitored at 254 nm in order to quantify the flavonoid quercetin.

2.5. 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 (124 mm NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 12 mM glucose, 1 mM $CaCl_2$, and 25 mM HEPES pH 7.4). Hippocampal slices were obtained according to Vinadé & Rodnight [14], briefly: a Mcilwain tissue chopper was used to obtain the slices (0.4 mm) that were separated and preincubated at 37°C for 30 min in microwell plates filled with HEPES saline (200 μL/slice). Subsequently, fresh medium was replaced (200 μL/slice) for control condition and treatments with Cdt (1, 5 and 10 μg/mL), HBE (100 μg/mL), HBE + Cdt, quercetin (10 μg/mL), and quercetin + Cdt and incubated for 1 hour (37°C).

2.6. Hippocampal Slices Viability. Immediately after incubation with 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) [15]. 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 = 550 nm) was measured in an ELISA reader equipment [16].

2.7. Phrenic Nerve-Diaphragm Preparation. Whole diaphragms along with the phrenic nerves were removed from mice killed by carbon dioxide (CO_2) and exsanguinated. Both hemidiaphragms were mounted essentially as described for dal Belo et al. [17]. The preparations were suspended under a constant tension of 5 g in a 5 mL organ bath containing aerated (95%O_2–5%CO_2) Tyrode solution (pH 7.4, 37°C) of the following composition (mM): NaCl 137, KCl 2.70, $CaCl_2$ 1.80, $MgCl_2$ 0.490, NaH_2PO_4 0.420, $NaHCO_3$ 11.9, and glucose 11.1. Supramaximal stimuli (0.1 Hz, 0.2 ms) delivered by a Grass S4 electronic stimulator (Grass Instrument Co., Quincy, MA, USA) were applied through electrodes placed around the motor nerve, corresponding to an indirect stimulation.

2.8. Statistical Analysis. The results were expressed as the mean ± SEM and were compared statistically using ANOVA for repeated measures. A P value < 0.05 indicated significance.

3. Results

HBE was shown to be rich in flavonoids derivatives such as kaempferol, quercetin, and quercetin glycosides (quercitrin, isoquercitrin, guaijaverin, and hyperoside) [13]. The selective extraction of polyphenol compounds in HBE resulted, after hydrolysis, in not less than 6,7% of total flavonoids, expressed as quercetin. Incubation of mouse phrenic nerve-diaphragm preparation (PND) with Tyrode solution did not induce alterations in basal muscle twitch tension during 120 min recordings (n = 5, Figure 1). When *Crotalus durissus terrificus* venom (Cdt, 10 μg/mL) was added to (PND) preparation there was an increase of 160% in the muscle twitch tension followed by an irreversible and complete neuromuscular blockade after 70 min (n = 5, Figure 1). Incubation of PND preparation with HBE (10 and 100 μg/mL) produced no alteration in the amplitude of muscle twitch tension (n = 5), during 120 min observation. However, when preparations were assayed with a mixture of HBE (50 μg/mL and 100 μg/mL) and Cdt venom (10 μg/mL) previously incubated

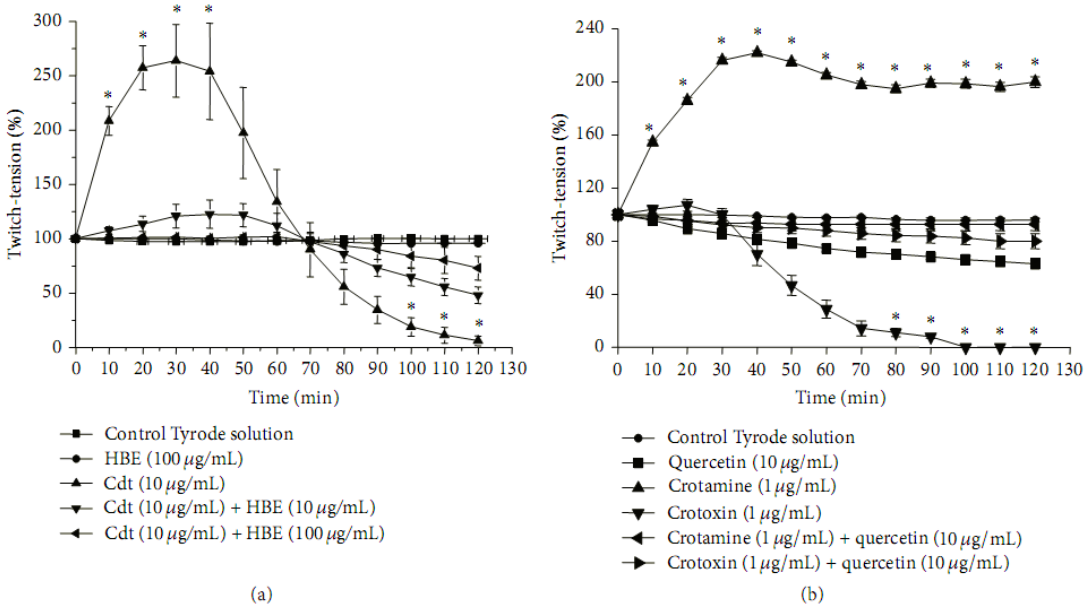

FIGURE 1: Neutralizing activity of *H. brasiliense* ethanolic plant extract (HBE) against Crotalic venom, crotoxin and crotamine at mouse phrenic nerve-diaphragm preparations. Panel (a) shows the inhibitory effect of HBE (10 and 100 μg/mL) against *C. d. terrificus* venom (10 μg/mL), crotoxin and crotamine. In Panel (b) effect of Quercetin (10 and 100 μg/mL) against Cdt (10 μg/mL), crotoxin (1 μg/mL), and crotamine (1 μg/mL). When HBE was applied alone in the organ-bath no alteration in the twitch-tension was observed. On both graphs control Tyrode solution lines show no alteration of normal nerve-muscle activity. The points on the graphs represent the mean ± S.E.M. of five experiments. On (b) note that quercetin mimicked the protective effect induced by HBE. HBE: *Hypericum brasiliense* standardized extract $^*P < 0.05$ compared to control Tyrode.

during 30 min at 37°C, the characteristic neuromuscular blockade was prevented in 75% with the highest concentration of the extract (Figure 1(a), $n = 5$, $P < 0.05$). The assay of the myotoxin crotamine (1 μg/mL) alone at PND preparations induced a significative increase of muscle twitch tension (~150%), that was maximum at 30 min ($P < 0.05$, $n = 6$, Figure 1(b)). On the contrary, the addition of the PLA_2 neurotoxin crotoxin isolated (1 μg/mL) at PND preparations caused a progressive and irreversible neuromuscular blockade during 120 min recordings ($P < 0.05$, $n = 6$). The assay of HBE (100 μg/mL) + crotamine (1 μg/mL) or crotoxin (1 μg/mL), previously incubated for 30 min at 37°C, inhibited 100% of the facilitatory actions induced by crotamine and 85% of the neuromuscular blockade caused by crotoxin (1 μg/mL), respectively, in 120 min recordings ($n = 5$, $P < 0.05$, data not shown). When quercetin (10 μg/mL) was incubated alone, there was a maximum decrease of muscle twitch tension of 20 ± 0.5% in 120 min recordings, although not significative (Figure 1(b), $P > 0.05$ compared to the control Tyrode). The addition of quercetin (10 μg/mL) with crotamine (1 μg/mL) or crotoxin (1 μg/mL) previously incubated for 30 min at 37°C showed a more potent antineurotoxic activity when compared to the HBE. This increased potency of quercetin compared to HBE must be due to a higher effective concentration of the flavonoid when compared to the whole extract (~7%). Quercetin was able to completely inhibit the facilitatory actions of crotamine (1 μg/mL) and decreased in 80 ± 5% the neuromuscular blockade induced by crotoxin (1 μg/mL) ($n = 5$, $P < 0.05$, Figure 1(b)).

The effect of HBE (100 μg/mL) or quercetin (10 μg/mL) alone was accessed at central nervous system (CNS) through hippocampal slices. In this set of experiments the cell viability was not modified after 1 h incubation with both vegetal extract and the pure flavonoid. On the other hand, the incubation of Cdt venom in doses of (1, 5, and 10 μg/mL) significantly decreased the cell viability (40 ± 3, 14 ± 1 and 28 ± 1%, $n = 3$, $P < 0.05$, resp.) (Figures 2(a) and 2(b)). The addition of HBE (100 μg/mL) with Cdt (10 μg/mL) to the slices produced a slight protection compared to the control Cdt ($n = 3$, $P < 0.05$) (Figure 2(a)). However, the blend of quercetin (10 μg/mL) and Cdt (1 μg/mL or 5 μg/mL), significantly inhibited the cell lysis showing a protection in the order of 46 ± 2% and 12 ± 1%, $n = 4$, $P < 0.05$, respectively (Figure 2(b)). The results in hippocampal slices confirm the HBE and quercetin potential role in the neuroprotection against Cdt poisoning. Therefore, the difference in potency between HBE and quercetin must also be related to the less amount of the flavonoid in the extract.

4. Discussion

In this work we described for the first time the effectiveness of the *H. brasiliense* extract (HBE) and its marked compound quercetin, against the neuromuscular paralysis induced by

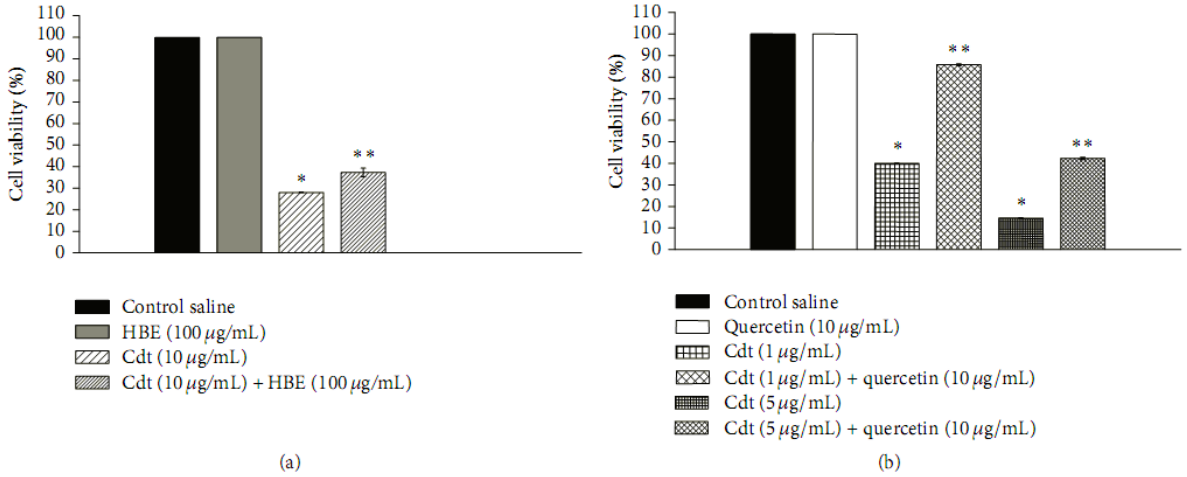

FIGURE 2: Effects of Cdt on the viability of hippocampal slices. (a) Hippocampal slices were incubated with HBE (100 μg/mL) in the presence or absence of Cdt (10 μg/mL) during 1 hour. (b) Hippocampal slices were incubated during 1 hour with Quercetin (10 μg/mL) in the presence or absence of Cdt (1 and 5 μg/mL). Cell viability was measured by MTT test. Values are expressed as % of control, which was defined as untreated slices (values are means ± S.E.M., $n = 4$). On (b) note that quercetin mimicked the HBE protective activity. HBE: *Hypericum brasiliense* standardized extract** $P < 0.05$ compared to control*.

Crotalus durissus terrificus snake venom (Cdt), crotoxin, and crotamine at mouse phrenic nerve-diaphragm preparations. Also, the effectiveness of HBE and quercetin was validated, to counteract the deleterious effects induced by *C. d. terrificus* venom, on cell viability of mouse brain slices. Crotalus venom induces neurotoxicity, coagulation disorders, systemic myotoxicity, and acute renal failure [18], with possible additional heart and liver damage [19]. This venom is a mixture of enzymes, toxins (crotoxin, crotamine, gyroxin, and convulxin), and several other peptides [19]. The characteristic pathophysiological pictures of neurotoxicity and systemic myotoxicity associated with *C. d. terrificus* envenomation are mainly related to the presence in the venom of crotoxin, a neurotoxic PLA_2 heterodimeric complex, which causes progressive paralysis, and in high concentrations myonecrosis [20, 21]. At nerve terminals, crotoxin induces triphasic alterations in the mean quantal content of transmitter release with a slow and progressive decrease of presynaptic release of the neurotransmitter acetylcholine that results in complete neuromuscular blockade [22, 23]. At mammal central nervous system, the injection of Cdt venom induces seizures [5], which is mainly associated with the presence of crotoxin [24]. At brain synaptosomes, crotoxin has also shown the ability of inhibiting L-glutamate and gamma aminobutyric acid (GABA) uptake [25]. Crotamine is the second major toxin in the Cdt venom; it is a basic, low molecular weight myotoxin devoid of PLA_2 activity [26], with a specific action on voltage-sensitive sodium channels of muscles [27] and brain cells [28].

Flavonoids are plant secondary metabolites that embrace a wealth of possibilities of hydrogen bonding arranged around a relatively small carbon skeleton, capable of interacting with molecular targets [29]. In the *H. brasiliense* extract, the flavonoid quercetin and its derivatives were shown to be the major secondary metabolites in the plant. Quercetin and several of its glycosides are the most often encountered flavonoids in anti-snake venom plants where *Albizia lebbeck*, *Achillea millefolium*, *Euphorbia hirta*, *Camellia sinensis*, and *Casearia sylvestris* are some examples. Flavonoids have been reported as snake venom phospholipase A_2 inhibitors [30].

Recent studies revealed that the treatment of the snake venom PLA_2 isoform from *Crotalus durissus cascavella* snake venom with the flavonoid quercetin produced a decrease in the pharmacological activity of the neurotoxin by inducing alterations in the secondary but not in tertiary structure composition of the molecule [31]. As discussed above, flavonoids have the ability of binding to biological polymers (e.g., enzyme inhibiting activities). Therefore, snake PLA_2 catalyzed the production of lysophospholipids and fatty acids that are involved in membrane damage [21]. We suggest that, in the case where biological activity is enzyme-dependent, the HBE antineurotoxic activity would involve the inactivation of PLA_2 activity by quercetin. However, the possibility that the HBE acts through a mechanistic intervention rather than an *in vitro* direct physical interaction with the venom is also a reasonable idea. This is likely to be the mode of action of many polyphenolic compounds found in plant extracts, which probably explains many of the "protective" effects of plant extracts when they are preincubated with venom before administration to the biological assay [32, 33].

Flavonoids derived from plants or tea extracts also affect acetylcholine release, muscle contraction, or neuromuscular junction activity [34]. In this regard, the muscle-type nicotinic acetylcholine receptor consists of $\alpha 1\beta 1\varepsilon$, in adult tissue [35]. It was found that quercetin inhibits the muscle type nicotinic acetylcholine receptor, by binding on the γ or ε subunities, which is a characteristic of a noncompetitive inhibitor [36]. Crotoxin also stabilizes the postsynaptic membrane of *Torpedo marmorata* by binding in non-ACh biding

sites [37]. Hence, these similarities in terms of binding sites would strengthen the hypothesis of a site-direct antagonism between quercetin and crotoxin at nerve terminals. In addition, quercetin actively participates in intracellular signaling, inhibiting phosphatidylinositol-3 kinase, protein kinase C, xanthine oxidase, and NADPH diaphorase [34]. In massive cellular insults like ischemia, involving metabolic failure, loss of Ca^{2+} homeostasis, and excitotoxicity, scavenger activity or one-target antioxidant mechanisms (NMDA receptor blockers, chain-breaking vitamin E, or pure scavenger molecules such as boldine) may fail to protect cells from free radical damage. Current explanation for the neuroprotective effect of quercetin is its antioxidant capacity and its ability to scavenge free radicals [34]. At moment there is no evidence that snake venoms induce cellular insults to increase free radicals in nerve terminals. However, the actions of Cdt venom on cell viability of brain slices is likely to be devoid to the presence of crotoxin and crotamine that ultimately account for the increase of excitatory neurotransmitters [22], resulting in excitotoxicity [38]. The decrease in neurotransmitter uptake by crotoxin is calcium independent [25], and quercetin potentiates neuronal excitability by increasing neuronal firing rates [39]. Ultimately, excitotoxicity is a result of synaptic dysfunction processes, which involves the excessive glutamate receptor activation and neuronal degeneration [38]. Based on the above considerations we suggest that the mechanism of the benefit of quercetin on snake venom-induced neuronal cellular death is complex and beyond the inhibition of presynaptic activity of snake PLA_2, and structural modifications, which may affect neurotransmitter uptake, involve the maintenance of neuronal mitochondrial transmembrane electric potential which would decrease the overstimulation of glutamate receptors [34]. However, in the case of crotamine, a direct inhibition of voltage-gated sodium channels by quercetin seems to be a coherent explanation [40].

Further investigation on *Hypericum brasiliense* isolated compounds will strengthen the understanding of its antiophidian activity. Preclinical assays, including safety assessment protocols, could also open the way towards therapeutic use of *Hypericum brasiliense* especially when neurotoxic venoms are involved.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Gildo Bernardo Leite for the excellence in technical assistance with mouse phrenic nerve-diaphragm preparations. Dr. Thais Posser for kind loan of MTT. This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundo de Apoio ao Ensino, à Pesquisa e Extensão (FAEPEX), and Coordenação de Aperfeiçoamento de Pessoal de nível Superior-CAPES Edital Toxinologia 063/2010.

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9 ANEXO:

9.1 Resultado e Discussão:

Após os resultados obtidos com HBE e quercetina em preparação musculo-nervo de diafragma de camundongo e tendo em vista o efeito convulsivante do Cdt (MELLO & CAVALHEIRO, 1989), foram feitos testes de neuroproteção com Riluzole sob o efeito do Cdt em fatias de hipocampo de camundongo. A figura 6 mostra que Riluzole, em todas as concentrações testadas, não altera a viabilidade celular *per se* e promove neuroproteção ao reverter os efeitos deletérios produzidos por Cdt em fatias de hipocampo de camundongo.

Segundo De Sarro (2000), o Riluzole exerce atividade anticonvulsivante em modelos de camundongo, interferindo com a neurotransmissão mediada pelo glicina/NMDA e o complexo do receptor AMPA/cainato. Portanto, Riluzole pode estar agindo via NMDA/AMPA tambem na proteção exercida contra o veneno de Cdt.

Figura 7 – Efeito Neuroprotetor do Riluzole em fatias de hipocampo incubadas com Cdt.

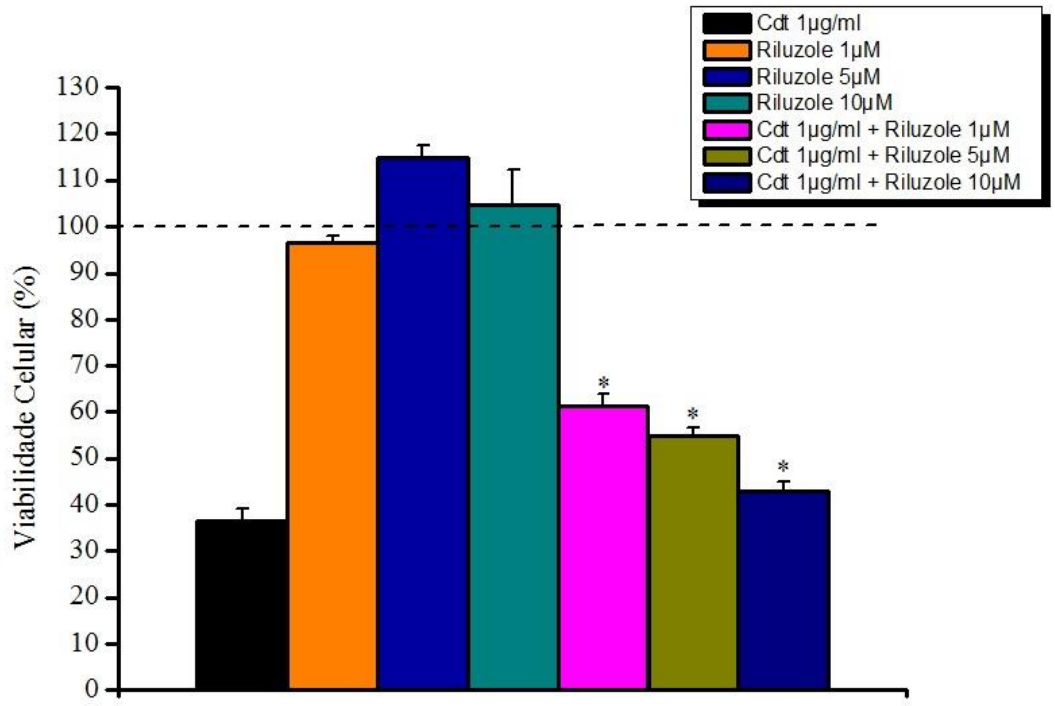

Figura 6: Gráfico sobre o efeito da neuroproteção de Riluzole contra veneno de Cdt em fatias de hipocampo de camundongo. Quando Riluzole (1, 5 e 10 µM) é aplicado sozinho não há alteração na viabilidade celular, assim como no controle com Hepes-salina. Os valores são expressos em percentual (%) em relação ao controle (linha pontilhada), o qual foi definido como fatias não tratadas, (médias ± SEM, n=4, ∗p<0.05, em relação ao controle Hepes-salina). Cdt: *Crotalus durissus terrificus.*

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