SARAGUACI HERNANDEZ DE OLIVEIRA E SILVA

Estudo Comparativo - Bioquímico e Imunofarmacológico- do Veneno Total, da Crotoxina e Suas Isoformas de Crotapotina e PLA_2, *de Crotalus durissus terrificus, C. d. ruruima, C. d. cascavella e C. d. collilineatus*

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Tese de Doutorado apresentada ao Curso de Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas UNICAMP - para obtenção do Título de doutor na área de Farmacologia.

Orientador: Profa. Dra. Léa Rodrigues-Simioni

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(Quarteto AVC)

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Seu amor em minha vida . Para que o mundo veja Cristo vive em mim

Quando o mundo vir que hoje sou de Cristo

Quando o mundo vir que Ele vive em mim

Então o amor, e a Sua paz irão resplandecer

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Pode o mundo vir que hoje sou de Cristo.

Pode o mundo vir que Ele vive em mim.

O Seu amor a paz irá reinar

Nos corações daqueles que não podem ver.

Este trabalho está dividido em três momentos, o primeiro trata-se de um estudo comparativo do efeito dos venenos brutos de *Crotalus durissus terrificus; C. d. ruruima; C. d. cascavella e C. d. collilineatus,* bem como da crotoxina e da crotamina sobre a junção neuromuscular.

O veneno *C. d. ruruima* foi purificado pela primeira vez através de HPLC em uma coluna Protein Pack SW 300, onde foi obtida a crotoxina e a crotamina (os demais venenos já foram purificados e os dados publicados). O estudo neurotóxico foi realizado em duas preparações nervo-frênico diafragma de camundongo e *biventer* cervicis de pintainho.

Dos experimentos realizados em mamíferos e aves obteve-se o efeito neurotóxico esperado nas concentrações de 10 μg/ml. Os venenos brutos e as crotoxinas das serpentes *C. d. terrificus e C. d. ruruima* em preparações de mamífero produziram aumento da amplitude seguido de bloqueio total da resposta contrátil, diferentemente dos venenos e das crotoxinas das serpentes *C. d. cascaavella e C. d. collilineatus,* que causaram apenas bloqueio total neuromuscular. Em preparações de aves os quatro venenos e crotoxinas estudados induziram bloqueio total, porém sem causar facilitação.

As crotaminas-positivas, estudadas em nervo-frenico diafragma de camundongo das serpentes *C. d. terrificus e C. d. ruruima*, na concentração de 10 μg/ml, causaram efeito facilitatório retornando à amplitude controle após 120 min de incubação. Ao recombinar a crotoxina 10 μg/ml e crotamina 5 μg/ml houve o aumento da amplitude seguido de bloqueio total da resposta contrátil. Nas preparações ensaiadas em aves, apresentou um efeito facilitatório fugaz seguido de bloqueio neuromuscular total.

No segundo momento, as isoformas de crotapotina e PLA_2 isoladas a partir da crotoxina dos venenos das serpentes de *C. d. terrificus, C. d. cascavella e C. d. collilineatus,* foram ensaiadas em mamíferos e aves nas concentrações de 10 μg/ml. As crotapotinas e PLA_2 isoladas não causaram diferenças significativas em relação ao controle em preparações de mamíferos. O mesmo não ocorrendo com as PLA_2 testadas em aves que causaram bloqueio neuromuscular, efeito este não esperado. Após a recombinação (5 μg/ml crotapotina) + (5 μg/ml PLA_2), o complexo crotoxina causou bloqueio neuromuscular total em ambas preparações.

Na terceita etapa deste trabalho, foram produzidos antivenenos específicos contra o veneno bruto e crotoxina do veneno *C. d ruruima* e PLA_2 do veneno de *C. d. collilineatus*. O título de anticorpos e a especificidade dos antivenenos produzidos foram avaliados por ELISA.

A neutralização da atividade neurotóxica foi avaliada em preparações *biventer* cervicis de pintainho. A capacidade neutralizante dos antivenenos produzidos em coelhos foi comparável ao soro anticrotálico comercial contra o veneno e crotoxina na proporção 1:1.

O veneno, a crotoxina e os antivenenos estudados induziram liberação de CK significativamente diferente do controle.

1. INTRODUÇÃO

O estudo dos venenos ofídicos é de grande importância sob vários aspectos. Deve-se ressaltar que a elucidação da fisiopatologia dos envenenamentos é fundamental para o avanço na terapia dos acidentes ofídicos. O estudo desses venenos vem contribuindo para o conhecimento de diversos processos fisiológicos e fisiopatológicos, destacando-se a estrutura e a função dos receptores nicotínicos, a cascata de coagulação sanguínea, a fibrinólise e o processo inflamatório.

A junção neuromuscular é o alvo de ação dos principais venenos ofídicos, em função dos seus componentes neurotóxicos e miotóxicos. Sendo assim, o estudo dos venenos ofídicos na área da Farmacologia da Junção neuromuscular constitui um importante e fértil campo de pesquisa.

Esta área teve seus primórdios com os trabalhos pioneiros de Vital Brazil (1901,1911), (Mineiro da Campana assim chamado) e seu grupo de pesquisa do Instituto Butantan, quando descreveu os efeitos dos venenos de animais peçonhentos na junção neuromuscular, causados em animais íntegros.

Em 1966, no Departamento de Farmacologia da UNICAMP, Oswaldo Vital Brazil e colaboradores retomaram os estudos sobre a farmacologia da crotoxina. Na seqüência em 1979 estudou a crotamina juntamente com seus colaboradores. A seguir, em 1981, em colaboração com Julia Prado Franceschi, relatou o efeito da convulxina.

Na década de 1980, por sugestão de Vital Brazil, Léa Rodrigues Simioni iniciou seus estudos com a peçonha de *Bothrops jararacussu*, culminando com o isolamento do pool IV posteriormente denominado BhTX (*Bothrops* toxina).

Graças ao esforço dessa pesquisadora, seu laboratório desenvolveu-se obtendo as condições necessárias para realização desta tese.

2. ACIDENTES OFÍDICOS

2.1. Aspectos gerais

Embora sejam de incidência mundial, os acidentes ofídicos ganham destaque em regiões tropicais, onde representam um sério problema de Saúde pública, devido a elevada

freqüência com que ocorrem e a morbidade e mortalidade que ocasionam (CHIPPAUX, 1998; PINHO & PEREIRA, 2001).

A ultima estimativa mundial, que data de 1998, aponta a ocorrência de cerca de 5 milhões de casos de acidentes ofídicos por ano, com aproximadamente 125.000 mortes; sendo os maiores índices de mortalidade encontrados em países da África e da Ásia (CHIPPAUX, 1998). Deve-se ressaltar, entretanto, que esses números certamente representam uma subestimativa, visto que são baseados apenas nos acidentes notificados.

No Brasil são registrados anualmente cerca de 20.000 casos de acidentes, com letalidade em torno de 0,45% (BRASIL – MINISTÉRIO DA SAÚDE, 2001). A maioria das notificações são procedentes das regiões meridionais do país, ocorrem no período de setembro a março, e estão relacionadas ao trabalho humano no campo. A faixa etária acometida varia de 15 a 49 anos de idade, sendo prevalente o sexo masculino , e os membros inferiores os mais atingidos.

Destacam-se as serpentes do gênero *Bothrops*, que representam 85% das notificações, e as do gênero *Crotalus*, pelo elevado índice de letalidade dos acidentes (1,85%), (PINHO & PEREIRA, 2001; BOCHNER & STRUCHINER, 2003).

Os venenos ofídicos são misturas complexas constituídas por proteínas (70 a 90%), em sua maioria toxinas ou enzimas tóxicas; podem conter peptídeos, carboidratos, lipídeos, aminas biogênica e componentes inorgânicos (DEVI, 1971). Os componentes inorgânicos mais freqüentes são Ca, Cu, Fe, K, Mg, Mn, Na, P, Co, Zn, exercendo função de manter estabilidade estrutural de determinadas proteínas ou agindo como catalisadores em reações enzimáticas (BJARNASON & FOX, 1988).

A proporção desses componentes é variável nos venenos das diferentes espécies de serpente, o que explica a diversidade da sintomatologia dos envenenamentos. Ocorre também uma variação na composição dos venenos dentro da mesma espécie de serpente, em função da estação do ano, da idade do réptil, sexo da serpente, e de sua distribuição geográfica.

As ações tóxicas dos venenos ofídicos podem atingir vários órgãos e sistemas, quer de modo direto, quer indiretamente, através da liberação de substâncias farmacologicamente ativas. As principais ações tóxicas dos venenos ofídicos são:

coagulante e hemorrágica - resultantes de componentes que atuam sobre os fatores da coagulação, ativando o fator X e a protrombina, estimulando, assim a formação de

trombina e, consequentemente, a formação de fibrina, ou atuando diretamente sobre fibrinogênio. O consumo de fibrinogênio promovido pelo veneno provoca ainda um estado de incuagubilidade sanguínea que intensifica as manifestações hemorrágicas produzidas pelas hemorraginas (NAHAS, *et al*., 1979).

anticoagulante – causadas por fatores ativadores da proteína C e inibidores da trombina e dos fatores IX e X da cascata de coagulação, entre outros mecanismos (SANO-MARTINS & SANTORO, 2003).

citotóxicas - determinada por fatores líticos diretos (cardiotoxinas), fosfolipases A_2 (PLA_2s) miotóxicas e por enzimas proteolíticas (GUTIERREZ & LOMONTE, 2003; KINI, 2003).

Vasculotóxica – causadas por fatores hemorrágicos denominados hemorraginas, que destroem a membrana basal dos capilares e posteriormente causam ruptura (BJARNASON & FOX, 1988).

neurotóxica – resultante de neurotoxinas, que causam o bloqueio da neurotransmissão, através de ações pré- e/ou pós-sinápticas, determinando a paralisia dos músculos esqueléticos (VITAL BRAZIL, 1980).

2.2. Acidentes crotálicos

As serpentes crotálicas no Brasil estão representadas apenas por uma espécie, a *Crotalus durissus*, distribuídas em cinco subespécies, *Crotalus durissus terrificus; C.d. ruruima; C.d. cascavella; C.d. collilineatus e C.d. marajoensis.* (SANTORO et al., 1999).

O veneno da subespécie *C.d.terrificus,* encontra-se melhor caracterizado, sendo considerado representativo do envenenamento crotálico de qualquer das subespécies catalogadas no país. Esta subespécie, como as demais, são encontradas nas zonas altas e secas da região sul oriental e meridional do país.

O veneno crotálico quase não produz reação no local, embora possa estar presente um discreto edema, na região acometida. A dor é pouco freqüente e quando ocorre não é intensa. Há relatos de parestesia local, que parece estar relacionada a ações antinociceptiva e analgésica do veneno (PICOLO & CURY, 2004).

O envenenamento crotálico é caracterizado por manifestações sistêmicas decorrentes das atividades neurotóxica, miotóxica e coagulante do veneno (AZEVEDO-MARQUES *et al*., 2003).

A neurotoxicidade é evidenciada após as primeiras seis horas do acidente por ptose palpebral, diplopia e flacidez da musculatura da face, caracterizando "fáceis neurotóxico", descrito por ROSENFELD (1971). Pode ocorrer dificuldade da deglutição e alterações do olfato e do paladar.

Há relatos de insuficiência respiratória aguda, interpretada como decorrente de paralisia da musculatura intercostal e/ou diafragmática (ROSENFELD, 1971).

A miotoxicidade manifesta-se pelo escurecimento da urina devido à eliminação da mioglobina, que configura a rabdomiólise. Em casos graves, a deposição de mioglobina nos túbulos renais pode evoluir para insuficiência renal aguda (AZAVEDO-MARQUES *et al*., 1982). A ação coagulante do veneno é evidenciada por alterações da coagulação sangüínea, com aumento do tempo de sangramento, ou mesmo incoagulabilidade sangüínea, em função do consumo de fibrinogênio (KAMIGUTI & SANO-MARTINS, 1995).

3. CROTOXINA

A crotoxina é uma proteína de 23 kDa isolado do veneno de *C.d. terrificus* (SLOTTA & FAENKEL-CONRAT, 1938) que representa cerca de 60 a 75% do peso seco total do veneno e constitui o principal determinante de sua toxicidade (VITAL BRAZIL, 1966; NASCIMENTO *et al*., 1996ª;). Apresenta potente atividade neurotóxica, promovendo bloqueio da transmissão neuromuscular e morte devido à paralisia respiratória em animais experimentais (VITAL BRAZIL, *et al.,* 1966; BREITHAUPT *et al*., 1974). Sua ação é predominantemente pré-sináptica, inibindo a liberação de acetilcolina pelas terminações nervosas motoras (VITAL BRAZIL & EXCEL, 1970; CHANG & LEE, 1977; HAWGOOD & SMITH, 1977).

Além da ação neurotóxica, a crotoxina exerce atividade miotóxica (AZEVEDO-MARQUES *et al*., 1982), hemolítica (ROSENFELD, 1971) e agregante plaquetária (LANDUCCI *et al.,* 1994).

A crotoxina é composta por uma subunidade básica com atividade PLA_2 e por uma subunidade ácida não enzimática, denominada crotapotina (HENDON & FRAENKEL-

CONRAT, 1971; BREITHAUPT *et al*., 1974; BREITHAUPT, 1976). As duas subunidades atuam em sinergismo, visto que, isoladamente, a PLA_2 é pouco tóxica, e a crotapotina é atóxica (RUBSAMEN *et al.,* 1971; BON *et al*., 1979). Sugere-se que a crotapotina atue como um carreador da PLA_2, evitando sua ligação a sitios não-especificos e, portanto, potencializando sua toxicidade (BON *et al*., 1979). Após a ligação da crotoxina a sítios específicos da membrana pré-sináptica e, provavelmente, também do músculo esquelético, o complexo se desfaz; a crotapotina é liberada e a PLA_2 exerce suas ações neurotóxicas e miotóxicas (DELÓT & BON, 1993). A inibição da atividade catalítica da subunidade PLA_2 reduz ou abole as atividades tóxicas da crotoxina (HAWGOOD & SMITH, 1977; JENG & FRAENKEL-CONRAT, 1978; MARLAS & BON, 1982; SOARES *et al*., 2001).

4.CROTAMINA

A crotamina, neurotoxina isolada a partir da peçonha de *Crotalus durissus terrificus* (cascavel sul-americana), foi a primeira proteína estudada no Brasil sob o aspecto bioquímico e farmacológico.

O quadro clínico mais evidente e característico por ela induzido é a intensa paralisação dos membros posteriores quando injetada por via i.p. ou i.v. em camundongos ou ratos.

A atividade biológica da crotamina e dos outros membros de sua subfamília dentro das miotoxinas é caracterizada pela sua ação sobre células musculares esqueléticas, causando danos ou a morte destas células. Esta ação se dá pela dilatação do retículo endoplasmático rugoso e sua conseqüente degeneração, conseqüência da alteração provocada pela proteína no transporte de íons de sódio através da membrana da célula muscular. O incremento da quantidade de Na+ no interior da célula provoca o inchaço do retículo sarcoplasmático pela excessiva retenção de água. A inabilidade da célula em regular seu equilíbrio osmótico leva à sua degradação (OWNBY, 1998).

Primeiramente isolado por Gonçalves e Vieira (GONCALVES & VIEIRA, 1950), possui peso molecular de 4,88 kDa, composta por 42 aminoácidos (LAURE, 1975). Possuindo 9 lisinas e 2 argininas, é extremamente básica, com pI=10,3 (GONÇALVES, 1956). A presença de 6 cisteínas interligadas por pontes dissulfeto confere à crotamina alta estabilidade conformacional (HAMPE *et al.*, 1978). A lista

completa dos aminoácidos e a seqüência primária da crotamina podem ser verificadas na Tabela 1.1.

Tabela 1. Seqüência primária da Crotamina e composição de aminoácidos.

1	10	20	30	40
YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG				

9 Lisinas	3 Serinas	2 Histidinas	1 Isoleucina
6 Cisteínas	2 Argininas	2 Triptofanos	1 Leucina
5 Glicinas	2 Aspartato	1 Glutamato	1 Metionina
3 Prolinas	2 Fenilalaninas	1 Glutamina	1 Tirosina

Como um polipeptídio pequeno, básico, mionecrótico e sem atividade enzimática, a crotamina faz parte de uma das três subfamílias de peptídeos encontradas em venenos de serpentes, chamadas miotoxinas (OWNBY, 1998). Estas proteínas apresentam uma alta homologia nas suas seqüências primárias, como visto na Tabela 2, que aliada à alta similaridade entre suas atividades biológicas, sugerem uma estrutura terciária comum a todas.

Tabela 2. Alinhamento das seqüências primárias de 8 proteínas pertencentes à família das miotoxinas. Posições onde são verificadas diferenças estão em destaque.

```
MYX1_CRODU   YKQCHKKGGH CFPKEKICLP PSSDFGKMDC RWRWKCCKKG SG
MYX1_CROVV   YKQCHKKGGH CFPKEKICIP PSSDLGKMDC RWKWKCCKKG SG
MYX_CROAD    YKRCHKKGGH CFPKTVICLP PSSDFGKMDC RWRWKCCKKG SVNNA
MYX2_CROVV   YKRCHKKEGH CFPKTVICLP PSSDFGKMDC RWKWKCCKKG SVNNA
MYXC_CROVH   YKRCHKKGGH CFPKTVICLP PSSDFGKMDC RWKWKCCKKG SVN
MYX1_CROVC   YKRCHKKEGH CFPKTVICLP PSSDFGKMDC RWKWKCCKKG SVN
MYX2_CROVC   YKRCHKKGGH CFPKEKICTP PSSDFGKMDC RWKWKCCKKG SVN
MYXC_CRODU   YKQCHKKGGH CFPKEKICLP PSSDFGKMDC RWRWKCCKKG SG
```

MYX1_CRODU: Crotamina de *Crotalus durissus terrificus*; MYX1_CROVV: Myotoxin a de *Crotalus veridis veridis*; MYX_CROAD: CAM-toxin de *Crotalus adamanteus*; MYX2_CROVV: Myotoxin II de *Crotalus veridis veridis*; MYXC_CROVH: Toxic peptide C de *Crotalus veridis helleri*; MYX1_CROVC: Myotoxin I de *Crotalus viridis concolor*; MYX2_CROVC: myotoxin II de *Crotalus viridis concolor* (Ownby, 1998).

Ao longo dos anos, a crotamina e suas similares têm sido estudadas em seus aspectos estruturais, funcionais e biofisicoquímicos, com resultados revistos por OWNBY (1998).

5. PROPRIEDADES IMUNOLÓGICAS DOS VENENOS OFÍDICOS

Os venenos ofídicos evocam diversos efeitos fisiopatológicos através de uma ou mais substâncias e, sobre este ponto de vista foram considerados como mosaico de antígenos (NICOLLE & RAPHAEL, 1925). Porém, quando se analisa a composição do veneno de famílias diferentes, este conceito de mosaico não se aplica, pois certos fragmentos podem ter uma estrutura relativamente similar ou não possuir qualquer semelhança (BAXTER & GALLICHIO, 1974). Deste modo, certos componentes são largamente distribuídos em várias espécies e outros são restritos a poucas espécies.

Em 1901, Vital Brazil demonstrou que os soros antiofídicos provenientes da Europa produzidos por Calmette não neutralizavam a ação dos venenos das serpentes brasileiras; demonstrou também que mesmo entre os venenos das serpentes encontradas na América do Sul o soro anticrotálico não era capaz de neutralizar a ação letal dos venenos de *Bothrops jararaca*, fato que o levou a descrever a especificidade dos soros antiofídicos. Apesar disso, muitos experimentos demonstram a presença de antígenos comuns nos venenos ofídicos de diferentes famílias, gêneros ou espécies.

LAMB & HANNA (1904), pela primeira vez evidenciaram, por reação de precipitação entre venenos de Naja naja e veneno de Echis carinatus, a presença de componentes comuns, condição esta confirmada mais tarde por uma série de experimentos com venenos de outras espécies ou gênero de ofídios. Com os avanços das técnicas que pudessem demonstrar as reações cruzadas entre os venenos, tais como: imunodifusão em gel de agarose (OUDIN, 1952), ELISA (ENGVALL & PERLMANN, 1972), muitos trabalhos demonstraram a composição ideal de antígenos para a produção de antivenenos (GRASSET *et al.*, 1956; THEAKSTON & REID, 1979), pois a presença ou não de componentes comuns no veneno de espécies do mesmo gênero faz com que o veneno de certas espécies sejam indispensáveis nas formulações antigênicas enquanto as outras se tornam redundantes.

Baseando-se nas possíveis reações cruzadas entre os venenos de serpentes existentes no Brasil, DIAS DA SILVA *et al*., (1989) contribuíram para a formulação do antígeno que é usado na imunização de eqüídeos para produção de soros antiofídicos.

Com a PLA_2 , que é um dos componentes da fração tóxica principal do veneno crotálico (crotoxina = crotapotina + Fosfolipase A_2) foram imunizados eqüinos cujo soros, neutralizaram a atividade letal do veneno crotálico integral, conforme teste em camundongos (HIGASHI *et al.*, 1989). Como demonstrado por SANTOS *et al.,* (1988) a

principal contribuição do estudo de HIGASHI *et al.*, (1989), foi a de possibilitar a indução da imunidade básica nos cavalos, os quais apresentaram reações locais ou sistêmicas menos intensas mesmo quando, posteriormente, receberam doses de veneno crotálico integral.

Portanto, a utilização das frações do veneno ou a formulação do antígeno a ser utilizado na produção de antivenenos é uma forma de melhorar a especificidade e a potência.

A crotoxina do veneno de *C.d. terrificus* é representativa de um grande número de neurotoxinas pré-sinápticas encontradas nos venenos de cascavéis. Um interesse considerável tem sido mostrado por essas toxinas a fim de conhecer detalhadamente seu mecanismo de ação e também entender melhor a liberação do neurotransmissor acetilcolina.

Anticorpos monoclonais e policlonais produzidos contra essas neurotoxinas tem sido uma ferramenta importante para que suas funções biológicas sejam melhor entendidas (KAISER & MIDDLEBROK, 1988).

Para o veneno *C. d. terrificus* já foi demonstrado que o antiveneno produzido contra a crotoxina dele isolada neutraliza os maiores efeitos letais do veneno (FREITAS *et al*., 1990; OSHIMA-FRANCO *et al*., 1999).

Antivenenos produzidos em coelhos contra o veneno total e crotoxina de *C.d.terrificus* e *C.d.cascavella* são melhores do que antivenenos eqüinos em neutralizar suas atividades neurotóxicas e miotóxicas (OSHIMA-FRANCO *et al.,* 1999; BEGHINI *et al.,* 2004).

Anticorpos policlonais também foram produzidos em coelhos contra as duas subunidades da crotoxina Anticorpos anti-PLA_2 neutralizaram a potencia letal da crotoxina e inibiram sua atividade enzimática (CHOUMET *et al*., 1989).

Muito pouco tem-se pesquisado sobre a atividade imunológica dos venenos, sabe-se, no entanto, que entre os fenômenos observados no sistema imune causados pelos venenos ofídicos, os que mais despertaram a atenção foram: atividade inflamatória e a imunossupressão induzida pelo Fator de Veneno de Cobra (Cobra Venom Factor – CVF). O CVF é uma proteína obtida de Naja-naja que atua sobre o componente C3 do sistema complemento (COCHRANE *et al*., 1970).

Os venenos da família Viperidae são altamente flogísticos, porém, grande parte dos estudos sobre resposta inflamatória aos venenos é baseada em relatos clínicos e os

trabalhos experimentais enfocam principalmente atividade edematogênica (SOUZA & SILVA, 1993).

As composições complexas dos venenos ofídicos, a especificidade e a existência de subespécie de serpentes determinaram a necessidade do conhecimento das suas relações antigênicas sobre o sistema imunológico dos animais produtores de soro, tendo em vista que os soros heterólogos consistem num único tratamento confiável dos envenenamentos provocados por esses répteis.

5.1. Soros Antivenenos

Os estudos sobre a atuação dos venenos no sistema imunológico são escassos e, ainda, não decisivos. Todavia, os venenos de serpentes podem atuar nos animais soro produtores de modo incerto e, por muitas vezes, não são obtidas respostas necessárias à produção de soros antiofídicos em concentrações desejadas.

No Brasil, Vital Brazil produziu os primeiros lotes de soro antiveneno descobrindo a especificidade em relação às espécies de serpentes. Com isso iniciou-se a produção de soro antibotrópico, anticrotálico e antiofídico (antibotrópico e crotálico). Estes soros eram produzidos de modo simplificado e o produto era administrado nos indivíduos acidentados sem nenhuma purificação (VITAL BRAZIL, 1987).

Com a evolução da química e da imunoquímica diversos métodos começaram a ser utilizados para o aprimoramento da obtenção de soros heterólogos, entre eles a purificação de imunoglobulinas com sulfato de amônio ($(NH_4)_2SO_4$) e a clivagem de imunoglobulina por pepsina (PEPIN *et al.,* 1995).

Atualmente os soros são produzidos em sistemas fechados e automatizados, levando em conta a utilização das boas práticas de produção preconizadas por órgãos internacionais de controle de medicamentos (RAW *et al*., 1991).

Mesmo assim as obtenções de títulos consideráveis de anticorpos não levam em conta metodologias científicas adequadas.

O Instituto Butantan vem, nos últimos 10 anos, pesquisando formas adequadas de imunização através de esquemas peculiares a cada tipo de veneno, modificações nas formulações de adjuvantes, metodologias que atenuem a toxicidade dos antígenos aumentando assim a vida útil dos animais imunizados e, consequentemente, a produção de

soros, purificação de venenos, buscando metodologias específicas, de baixo custo ou de custo coerente ao beneficio, como por exemplo: identificação e seleção de cavalos, bons produtores de anticorpos a determinados imunógenos, toxinas ou venenos.

Os adjuvantes utilizados na hiperimunização de cavalos com bons resultados são Adjuvante de Freud Completo e Incompleto, que são administrados respectivamente no primeiro e segundo inóculos e o alginato de sódio ou hidróxido de alumínio nos demais (BARRAVIERA & PERAÇOLI, 1994; RUCAVADO *et a*l., 1996).

Devido a algumas reações adversas causadas por estes adjuvantes, tem-se realizado esforços no sentido de identificar novos adjuvantes eficientes para a produção de antivenenos (RUCAVADO *et al*., 1996).

O maior problema enfrentado na produção do soro antiofídico de modo geral, está na qualidade da matéria prima, especificamente no título do plasma sangüíneo a ser purificado. É de conhecimento dos laboratórios produtores que rendimentos acima de 40% são raros, pois os métodos de purificação, a sensibilidade dos IgG aos tratamentos e as perdas mecânicas inerentes ao processo fazem com que os rendimentos sejam relativamente baixos . Desse modo, é perfeitamente coerente que, para obtermos um produto final de boa qualidade, ou seja, com bons títulos, baixa proteína e sem formação de agregados protéicos é necessário que os plasmas tenham excelentes títulos.

2. OBJETIVOS

⇨Fracionamento do veneno da serpente *C.d ruruima* em HPLC fase reversa.

⇨ Comparar atividade farmacológica dos venenos bruto, crotoxina e crotamina das serpentes *C. d. terrificus, C. d.ruruima, C. d. cascavella, C. d. collilineatu*. em preparações isoladas nervo-frênico diafragma de camundongo e *biventer cervicis* de pintainho

⇨ Comparar atividade neurotóxica das isoformas de crotapotina e PLA_2 isoladas e recombinadas formando o complexo crotoxínico das serpentes *C. d. terrificus, C. d. cascavella e C. d. collilineatus*, utilizando as preparações de aves e mamíferos.

⇨ Avaliar a capacidade neutralizante de AV comercial e específico sobre a atividade neurotóxica e miotóxica do veneno bruto e crotoxina da serpente *C. d. ruruima,* utilizando preparação de ave.

⇨ Avaliar a capacidade neutralizante da PLA_2 *C. d. collilineatus* contra o veneno e crotoxina da serpente *C. d. ruruima*.

Comparison of the neurotoxic effects of *Crotalus durissus terrificus*, *C. d. ruruima, C. d. cascavella* and *C. d. collilineatus* venoms and their crotoxin homologs

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Running title: Comparative neurotoxicity of *C. durissus* venoms

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Abstract

Several subspecies of the tropical rattlesnake, *Crotalus durissus*, occur in Brazil. In this work, we compared the neuromuscular activities of *Crotalus durissus terrificus*, *C. d. ruruima*, *C. d. cascavella*, and *C. d. collilineatus* venoms and their crotoxin and crotamine homologs in indirectly stimulated mouse phrenic nerve-diaphragm and chick biventer cervicis preparations. All of the venoms and their crotoxin homologs (10 g/ml each) caused complete neuromuscular blockade within 120 min in both preparations. However, only *C. d. terrificus* and *C. d. ruruuima* venoms (10 g/ml each) produced an initial increase in twitch-tension (187 ± 40% and 167 ± 29%, respectively; mean±S.E.M., n=7 and 6, respectively) in mouse preparations. Crotamine (10 g/ml) from *C. d. terrificus* and *C. d. ruruima* venoms (the *C. d. cascavella* and *C. d. collilineatus* venoms contained no crotamine) caused an initial facilitation (167 ± 38%, n=8, and 163± 23%, n=11, respectively, in mouse preparations, and 37 ± 12% and 21 ± 7%, respectively, in chick preparations, n=5 each) that, in mouse preparations, was not significantly affected by the presence of crotoxin. In mouse and chick preparations, crotoxin from the four subspecies caused complete, irreversible neuromuscular blockade within 60-70 min and 15-30 min, respectively. None of the crude venoms or the two crotoxin or crotamine homologs inhibited ACh- or KCl-induced contractures in chick preparations. These results show that the venoms of these four subspecies of *C. durissus* and the crotoxin and crotamine homologs of *C. d. terrificus* and *C. d. ruruima* had similar neuromuscular activities.

Keywords: Chick biventer cervicis; *Crotalus durissus* subspecies; Crotamine; Crotoxin; Mouse phrenic nerve diaphragm; Neuromuscular blockade; Neurotransmission; Rattlesnake venom.

1. Introduction

In Brazil, envenomations caused by rattlesnakes (*Crotalus durissus* subspecies) account for 7-40% of bites by venomous snakes, depending on the region of the country (Barravieira, 1994; Schavartsman, 1992; Sgarbi et al., 1995; Araujo et al., 2003). The fatality rate of bites by *Crotalus durissus terrificus* can reach 72% in cases not treated with antivenom, but decreases to 11% in cases treated with antivenom (Amaral et al., 1986). The main effects of envenoming by Brazilian *C. durissus* ssp. are neurotoxicity (with accompanying muscular weakness), coagulopathy, systemic myotoxicity, characterized by rhabdomyolysis, and renal failure (Azevedo-Marques et al., 1985, 1986; Amaral et al., 1986; Brazil, 1990).

Several toxins have been purified from *C. durissus* ssp. venoms, including convulxin (Prado-Franceschi and Vital Brazil, 1981), gyroxin (Barrio, 1961), crotamine (Gonçalves and Vieira, 1950; Gonçalvez and Arantes, 1956) and crotoxin (Slotta and Fraenkel-Conrat, 1938), which was the first snake toxin to be obtained in crystalline form. Crotoxin, a β-neurotoxin, is the major component of *C. d. terrificus* venom and consists of a basic, weakly toxic phospholipase (PLA_2), referred to as component B or crotactin, and an acidic, nontoxic, nonenzymatic subunit known as component A or crotapotin (Rubsamen et al., 1971; Hendon and Fraenkel-Conrat, 1971; Breithaupt et al., 1974). The subunits are not covalently linked and their dissociation abolishes the neurotoxicity of the complex, although this can be restored by reassociation of the complex. Component B is a single polypeptide chain whereas crotapotin is composed of three polypeptide chains linked by disulfide bridges (Habermann and Breithaupt, 1978; Fraenkel-Conrat et al., 1980; Aird et al., 1986). Both components of the crotoxin complex have amino acid sequences similar to those of snake venom and pancreatic PLA_2, but crotapotin lacks the region involved in binding the phospholipid substrate (Aird et al., 1985).

Vellard (1937) reported that the physiopathological effects of *Crotalus durissus* venom varied according to the geographic origin of the venom. Subsequently, Gonçalves and Arantes (1956) and Schenberg (1959) showed that there were variations in the content of crotamine in *C. d. terrificus* venom. This small, 42-amino-acid, basic (pI 10.3) myotoxin is related to a group of myotoxin-a-like proteins that are widely distributed in rattlesnake venoms (Bober et al., 1988; Ownby, 1998). The content of crotamine in *C. d. terrificus* venom varies geographically within and among subspecies (Schenberg, 1959; Toyama et al., 2005) and accounts for approximately 10-22% of the venom weight. Crotamine is absent from populations of *C. d. terrificus* in northern and eastern Brazil, but is present in northwestern São Paulo State and adjacent areas of the states of Paraná and Minas Gerais; populations with and without crotamine also occur in the northeastern state of Ceará (Schenberg, 1959). More recent studies have confirmed the individual variation in the composition of *C. d. terrificus* (Francischetti et al., 2000; Magro et al., 2001) and *C. d. ruruima* (Dos-Santos et al., 2005) venoms.

In Brazil, *Crotalus durissus* is represented by at least five subspecies, namely, *C. d. terrificus*, *C. d. ruruima*, *C. d. cascavella*, *C. d. collilineatus* and *C. d. trigonicus* (Amaral, 1978; Campbell and Lamar, 1989; Soerensen, 1990; Melgarejo, 2003). The venoms of *C. d. terrificus, C. d. cascavella,* and *C. d. collilineatus* have a similar profile of biological activities, although the paw edema-inducing activity (Rangel-Santos et al. de 2004), electrophoretic profile, phospholipase A_2 activity and inorganic element content of *C. d. cascavella* venom shows minor quantitative differences compared to the other venoms (Santoro et al., 1999); the neuromuscular activity of venoms from these subspecies was not investigated by these authors

In this work, we compared the neuromuscular actions of *C. d. terrificus*, *C. d. ruruima*, *C. d. cascavella* and *C. d. collilineatus* venoms and their crotoxin and crotamine homologs in mouse phrenic nerve-diaphragm and chick biventer cervicis muscle preparations.

2. Material and Methods

2.1. Reagents and venoms

Solvents (HPLC grade) and other reagents were purchased from Sigma and Aldrich Chemical Co. (St. Louis, MO, USA). *Crotalus durissus terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms was collected from adult specimens of both sexes captured in the state of São Paulo; *C. d. ruruima* venom was from specimens captured in the state Roraima.

2.2. Purification of crotoxin and crotamine homologs by molecular exclusion HPLC

Crotoxin and crotamine were purified from *C. d. terrificus* venom as described elsewhere (Toyama et al., 2000). Crotoxin from *C. d. cascavella* and *C. d. collilineatus* was purified as described by Beghini et al. (2000) and Ponce-Soto et al. (2002), respectively. Crotoxin and crotamine were purified from *C. d. ruruima* venom using a combination of the procedures described for other *C. durissus* ssp. *Crotalus d. ruruima* venom (20 mg) was loaded onto a Protein-Pack 300 SW column (0.78 cm x 30 cm) and eluted with 0.25 M ammonium bicarbonate, pH 7.9, at a flow rate of 3 ml/min. The elution profile was monitored at 280 nm using a Waters UV/visible detector model 490 and fractions were collected using a Foxy 200 automatic fraction collector. The chromatographic runs were recorded using a Waters model 746 recorder fitted with a data module-dual channel integrator.

2.3. Electrophoresis

Tricine-PAGE in a discontinuons gel and buffer system (Schagger and von Jagow, 1987) was used to estimate the molecular mass of the proteins.

2.4. Mouse phrenic nerve-diaphragm preparation

Adult male Swiss white mice (28-35 g) were supplied by the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP). The phrenic nerve and diaphragm were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrified by exsanguination. The nerve-muscle preparation was mounted as described by Bülbring (1946). Hemidiaphragms and phrenic nerves were mounted in 5 ml tissue baths containing aerated (95%O_2 - 5%CO_2) Tyrode solution (pH 7.4, 37°C) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1. The preparations were mounted on an electrode that allowed indirect (supramaximal voltage, 0.1 Hz, 0.2 ms) and direct (50 V, 0.1 Hz, 2 ms) stimulation of the muscle with impulses delivered from a Grass S4 stimulator (Grass Instruments, Quincy, MA, USA). The resulting muscle tension was recorded using a force displacement-transducer (BG 25 GM, Kulite Semiconductor Products, Inc., Leonia, NJ, USA) coupled to a Gould RS 3400 recorder (Gould Inc., Cleveland, OH, USA). The preparation was allowed to stabilize for at least 15 min before the addition of venom (10 μg/ml), crotoxin (10 μg/ml) or crotamine (10 μg/ml). When crotoxin and crotamine were tested together, the concentration of each toxin was 5 g/ml.

2.5. Chick biventer cervicis preparation

Male chicks (4-8 days old) were killed with ether and the biventer cervicis muscle was removed (Ginsborg and Warriner, 1960) and mounted under a resting tension of 1 g in a 4 ml

organ bath containing aerated (95%O_2 - 5%CO_2) Krebs solution (pH 7.5, 37^{o}C) of the following composition (in mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, NaHCO 25.0 and glucose 11.65. A bipolar platinum ring electrode was placed around the tendon and indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz, 0.2 ms). Muscle contractions and contractures were recorded with a BG-10 GM Kulite displacement transducer coupled to a Gould RS 3400 recorder. Contractures to exogenously applied acetylcholine (ACh, 110 μM for 60 s) and KCl (20 mM for 120 s) were obtained in the absence of nerve stimulation prior to the addition of toxin and at the end of the experiment. The preparation was allowed to stabilize for at least 15 min before the addition of venom, crotoxin or crotamine, as described above for the mouse preparation.

2.6. Statistical analysis

The results were expressed as the mean ± S.E.M. and statistical comparisons were done using Student's t-test, with a value of $p < 0.05$ indicating significance.

3. Results

3.1. Fractionation of Crotalus durissus ruruima venom

The fractionation of *C. d. ruruima* venom by molecular exclusion HPLC resulted in four main peaks that corresponded to convulxin (peak I), gyroxin (peak II), crotoxin (peak III) and crotamine (peak IV) (Fig. 1). The pharmacological activity of the latter two peaks was assayed in mouse and chick nerve-muscle preparations. Tricine SDS-PAGE showed that *C. d. ruruima* crotoxin had two major bands with molecular masses of 15 and 26 kDa, whereas the estimated molecular mass for crotamine was 9 kDa (Fig. 1A).

3.2. Neuromuscular activity in mouse phrenic nerve-diaphragm preparations

A single concentration (10 μg/ml) of each venom and their crotoxin homologs was tested in indirectly stimulated mouse phrenic nerve-diaphragm preparations. This concentration was chosen based on preliminary experiments which showed that it produced neuromuscular blockade within a reasonable time scale (<120 min). All of the venoms caused complete, irreversible neuromuscular blockade of the preparations within <80 min (Fig. 2). Table 1 shows the time required for 50% blockade by the venoms and crotoxin. Prior to causing blockade, the venoms of *C. d. terrificus* and *C. d. ruruima* produced an initial increase (within 10 min of application) in the twitch-tension amplitude of 144 ± 25% and 83 ± 22%, respectively, that reached 187 ± 40% after 16 ± 1.4 min and 167 ± 29% after 30 ± 6 min, respectively. No similar increase in twitch-tension was seen with *C. d. cascavella* or *C. d. collilineatus* venoms (Fig. 2A).

All of the crotoxin homologs (10 g/ml) produced complete neuromuscular blockade without a significant increase in twitch-tension (Table 1 and Fig. 2B). Crotoxin (10 g/ml) from *C. d. terrificus* and *C. d. ruruima* venom caused significant facilitation that reached 167 ± 37% at 18 ± 2 min and 163 ± 23% at 19 ± 4 min, respectively, followed by a decline in the amplitude to control levels (Fig. 3A).

A combination of crotoxin (5 μg/ml) and crotamine (5 μg/ml) from *C. d. terrificus* venom caused an initial facilitation that reached 136 ± 11% at 12 ± 3 min, whereas a combination of the corresponding toxins from *C. d. ruruima* produced a facilitation of 215 ± 26% at 11.2 ± 1.2 min, followed, in both cases, by complete, irreversible blockade of the contractile responses (Fig. 3B). Table 2 shows the time for 50% blockade and the percentage increase in twitch tension caused by crotoxin and crotamine from the different venoms.

3.3. Neuromuscular activity in chick biventer cervicis preparations

As with the mouse preparations, a single concentration (10 g/ml) of the venoms and crotoxin homologs produced complete neuromuscular blockade in chick biventer cervicis preparations, but without the initial increase in twitch tension seen for *C. d. terrificus* and *C. d. ruruuima* venoms in the former preparations (Fig. 4). Table 1 shows the time required for 50% blockade by the venoms and crotoxin. The responses to ACh and KCl were unaffected after complete blockade of the twitch-tension response (data not shown).

Crotamine from *C. d. terrificus* and *C. d. ruru*ima venoms caused a discreet but significant increase in twitch-tension amplitude that reached 37 ± 12% and 21 ± 7%, respectively (both at 10 min), followed by a decline in twitch amplitude to control levels and a slight decrease (17%) in the contractile response of the muscle after 120 min (Fig. 5). The combination of crotoxin and crotamine did not cause the facilitation seen in mouse preparations, but only the neuromuscular blockade characteristic of crotoxin and venoms (Fig. 5). Table 3 shows the time for 50% blockade and the percentage increase in twitch tension.

4. Discussion

Crotalus d. terrificus venom contains a variety of peptides, toxins (convulxin, gyroxin, crotoxin and crotamine) and enzymes (tissue kallikrein-like activity, thrombin-like enzyme, phosphodiesterase, 5'-nucleotidase and L-amino acid oxidase) (Bercovici et al., 1987). Crotoxin, the main toxin of this venom, exerts its lethal effect by blocking neurotransmission at the neuromuscular junction through a triphasic mechanism that involves mainly a presynaptic action (Vital Brazil and Excell, 1970; Hawgood and Smith, 1977; Rodrigues-Simioni et al., 1990).

The results of this study show that the venoms of the four subspecies and their crotoxin homologs studied here produced similar neuromuscular blockade in mouse phrenic nerve-diaphragm preparations. However, the venoms of *C. d. cascavella* and *C. d. collilineatus* did not cause the typical initial increase in the twitch-tension amplitude seen with *C. d. terrificus* and *C. d. ruruima* venoms before the onset of neuromuscular blockade. Since crotoxin did not increase the amplitude of indirectly elicited twitches, we attributed this facilitatory response to the absence of crotamine in these venoms (Santoro et al., 1999; Beghini et al., 2000; Ponce-Soto et al., 2002). *Crotalus d. ruruima* venom, which is crotamine-positive, increased the twitch-tension amplitude to a similar extent to that of *C. d. terrificus* venom and crotoxin.

Molecular exclusion chromatography by HPLC Existem diferenças bioquímicas entre os venenos totais e as crotoxinas de *Crotalus durissus terrificus, C. d. ruruima*, com as *C. d cascavella e C. d collilineatus.* Análise cromatográfica em dos venenos *C. d. collilineatus e C. d.cascavella*, mostram a presença das frações I, II, III e uma nova fração IV denominada Inter-CRO, contudo mostra a ausência de crotamina, quanto a *C. d terrificus* mostra a presença de sete frações (I), (II), III (que é nova), crotoxina (IV) fração V (Inter-CRO) fração VI (novas frações), e crotamina (VII). (Hernandez-Oliveira, et al., 2005, Beghini et al., 2000 e Ponce Soto et al., 2002). Laure (1990), mostrou um perfil cromatográfico com a presença de uma fração denominada Inter-CRO, eluída entre o complexo crotoxina e crotamina, sendo considerada uma iso-crotoxina, o que vem corroborar com nossos achados e testados em nosso laboratório evidencio-se uma nova fração causando facilitação da resposta contrátil (dados não mostrado).

As diferenças encontradas na crotoxina de *Crotalus durissus terrificus,* Reverse-Phase HPLC, foram encontrados duas isoformas de crotapotina (componente A) e três de PLA_2 (componente B), o mesmo não ocorrendo com o veneno de *C. d. cascavella* com a mesma

metodologia empregada foram encontrados quatro frações de crotapotina e uma de PLA_2 e de *C. d. collilineatus* duas de crotapotinas e uma de PLA_2 e para *C. d. ruruima* foram encontrados duas isoform de crotapotin e duas de PLA_2 (dados não mostrados). When studied activity PLA_2 gifts in the complete venom of these serpents and compared with the PLA2 of *C. d. terrificus* one observed differences in speed terms, as well as the proper characteristics of kinetic enzymatic (Toyama et al., 2000, Hernandez-Oliveira, et al., 2005, Beghini et al., 2000 and Ponce Soto et al., 2002). Crotapotin acts as a chaperon in the crotoxin complex by preventing non-specific binding of the PLA_2, thereby potentiating its toxicity (Bon et al., 1979). Hence, the stability of the interaction between the PLA_2 and crotapotin plays a major role in the toxicity of crotoxin (Faure et al., 1993).

The presented pharmacology effect in the behavior of these venoms through the miographic registers observed in such a way in bird as in mammal, could be related to the number of components of the crotoxinic complex (PLA_2 and crotapotin) of each studied venom and the presence of crotamin in the total venom of *C. d terrificus* and *C. d ruruima*, and absence in the venom of *C. d. cascavella* and *C. d collilineatus*, what it inside demonstrates to a strong indication of the importance of the crotoxin of the venom of *C. d. durissus* being the responsible main component for the pharmacology effect. The absence of crotamine in *C. d. cascavella* and *C. d collilineatus* also was observed by Santoro et al., 1999. This is the first report to describe the neuromuscular action of *C. d. ruruima* venom and its crotoxin and crotamine. The elution profile of *C. d. ruruima* venom after molecular exclusion chromatography indicated that crotoxin was the main component (as in other Brazilian subspecies of *C. durissus*) and accounted for 60% of the venom dry weight. Crotamine was the second major component. Crotamine isoforms have recently been reported to produce a facilitatory effect that increased the twitch-tension response

of mouse preparations by 230-300%, after a 120 min incubation (Toyama et al., 2003). As shown here, *C. d. terrificus* and *C. d. ruruima* venoms and their corresponding crotamine (with or without crotoxin) produced pronounced facilitation before neuromuscular blockade.

In chick biventer cervicis preparations, the venoms and their crotoxin homologs produced neuromuscular blockade, whereas crotamine did not block the twitch-tension responses at the concentration used but produced an initial augmentation in the twitch amplitude. Since crotoxin is much more active in chick preparations than in mouse preparations, the strong neuromuscular action of crotoxin probably masked the facilitatory action of crotamine in the former preparations.

The neurotoxic and myotoxic effects of venoms and toxins can be conveniently studied in chick biventer cervicis preparations. Presynaptically active neurotoxins abolish nerve-evoked twitches without affecting responses to cholinoceptor agonists, whereas myotoxic components reduce the responses of chick muscle to exogenous K^+ and/or produce contractures in the muscle (Harvey et al, 1994). In the present study, neither crotoxin, which inhibits neurotransmitter release presynaptically (Vital Brazil and Excell, 1970; Hawgood and Smith, 1977), nor crotamine, which is myonecrotic and causes skeletal muscle fiber vacuolation (Cameron and Tu, 1978; Gutierrez and Cerdas, 1984) altered the responses to ACh or KCl.

In conclusion, the results described here indicate that the venoms of the four *C. durissus* subspecies and their crotoxin and crotamine homologs had similar actions in avian and mammalian nerve-muscle preparations. The main difference observed, i.e., the inability of *C. d. cascavella* and *C. d. collilineatus* venoms to induce muscle facilitation, was attributable to the absence of crotamine in these venoms. The immunological similarity previously reported for at

least three of these venoms (Santoro et al., 1999) suggests that the neuromuscular activity seen here would be neutralized by antivenom raised against *C. d. terrificus* venom.

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Table 1. Time (min) required for 50% blockade in mouse phrenic nerve-diaphragm (PND) and chick biventer cervicis (BC) preparations incubated with *C. d. terrificus* (Cdt), *C. d. ruruima* (Cdr), *C. d. cascavella* (Cdc) and *C. d. collilineatus* (Cdcoll) venoms and their respective crotoxins.

Venom or toxin (10 g/ml)	PND	n	BC	n
C. d. terrificus	63 ± 8	7	16 ± 1	8
C. d. ruruima	78 ± 10	6	35 ± 2	6
C. d. cascavella	39 ± 2	5	32 ± 4	7
C. d. collilineatus	62 ± 1	6	21 ± 13	8
Crotoxin (Cdt)	64 ± 5	6	15 ± 2	4
Crotoxin (Cdr)	69 ± 3	5	24 ± 3	7
Crotoxin (Cdc)	37 ± 2	5	23 ± 2	6
Crotoxin (Cdcoll)	37 ± 4	6	20 ± 1	5

The values (in min) are the mean ± SEM of the number of experiments (n) shown.

Table 2. Neuromuscular activity of crotamine alone and in combination with crotoxin in mouse phrenic nerve-diaphragm preparations.

Toxin	Facilitation (maximum, %)	Time (min)	Time for 50% blockade (min)	n
Crotamine (Cdt)	167 ± 37	18.0 ± 2.0	----	8
Crotamine (Cdr)	163 ± 23	19.4 ± 4.0	----	11
Crotamine + crotoxin (Cdt)	136 ± 11	12.0 ± 3.0	38 ± 8	7
Crotamine + crotoxin (Cdr)	215 ± 26	11.2 ± 1.2	40 ± 5	9

Cdt – *C. d. terrificus*, Cdr – *C. d. ruruima*. Crotamine concentrations: 10 g/ml when alone, and 5 g/ml in combination with crotoxin (5 g/ml). The values are the mean ± SEM of the number of experiments (n) shown.

Table 3. Neuromuscular activity of crotamine alone and in combination with crotoxin in chick biventer cervicis preparations.

Sample	Facilitation (maximum, %)	Time (min)	Time for 50% blockade (min)	n
Crotamine (Cdt)	37 ± 12	6 ± 2	----	5
Crotamine (Cdr)	21 ± 7	14 ± 1	----	5
Crotamine + crotoxin (Cdt)	----	----	16.5 ± 2.0	4
Crotamine + crotoxin (Cdr)	----	----	33.5 ± 1.2	4

Cdt – *C. d. terrificus*, Cdr – *C. d. ruruima*. Crotamine concentrations: 10 g/ml when alone, and 5 g/ml in combination with crotoxin (5 g/ml). The values are the mean ± SEM of the number of experiments (n) shown.

Figure legends

Fig. 1. (A) Elution profile of *C. d. ruruima* venom by HPLC molecular exclusion chromatography. Cvx – convulxin, Crot – crotamine, Crtx – crotoxin, Gyr – gyroxin, V – unidentified toxin. See Methods for further details. (B) Electrophoretic profiles of *C. d. ruruima* venom, crotoxin and crotamine in Tricine SDS-PAGE. Mk, molecular mass markers (values in kDa).

Fig. 2. Neuromuscular blockade produced by (A) *C. d. terrificus, C. d. ruruima, C. d. cascavella* and *C. d. collilineatus* venoms and (B) their corresponding crotoxins (10 μg/ml in all cases) in mouse phrenic nerve-diaphragm preparations. Each point represents the mean ± S.E.M. of 4-7 (A) and 4-6 (B) experiments.

Fig. 3. Neuromuscular blockade produced by (A) crotamine (10 μg/ml) from *C. d. terrificus* and *C. d. ruruima* venoms alone and (B) crotoxin (10 μg/ml) alone or in combination with crotamine (5 μg/ml for each toxin) in mouse phrenic nerve-diaphragm preparations. Each point represents the mean ± S.E.M. of 4-11 experiments.

Fig. 4. Neuromuscular blockade produced by (A) *C. d. terrificus, C. d. ruruima, C. d. cascavella* and *C. d. collilineatus* venoms and (B) their corresponding crotoxins (10 μg/ml in all cases) in chick biventer cervicis preparations. Each point represents the mean ± S.E.M. of 4-8 experiments.

Fig. 5. Neuromuscular blockade produced by (A) crotamine (10 μg/ml) alone and (B) crotamine (5 μg/ml) + crotoxin (10 μg/ml) from *C. d. terrificus* or *C. d. ruruima* venoms in chick biventer cervicis preparations. Each point represents the mean ± S.E.M. of 4-5 experiments.

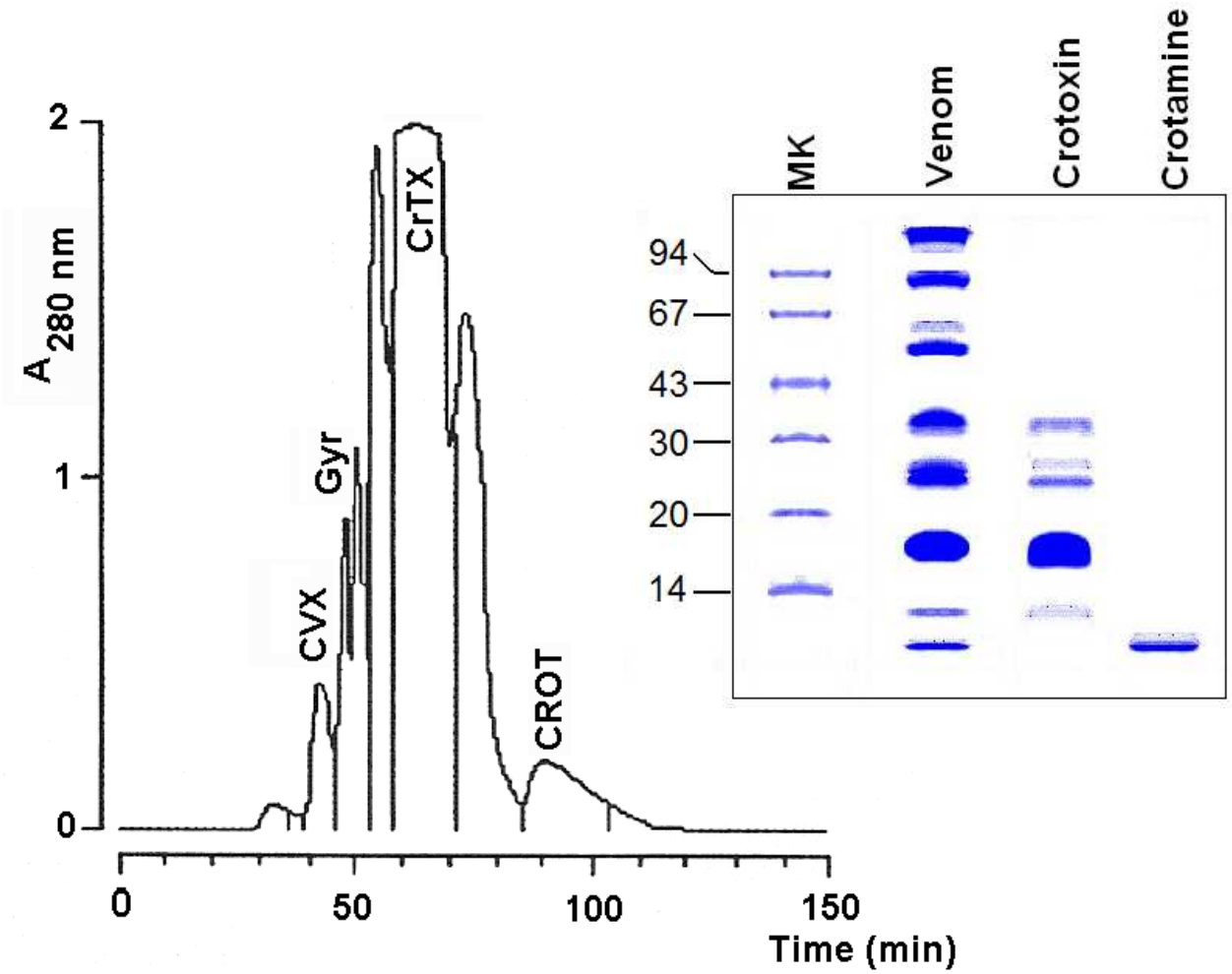


Fig. 1. Hernandez-Oliveira et al. – Comparison of the neurotoxic effects of *Crotalus durissus terrificus*.....

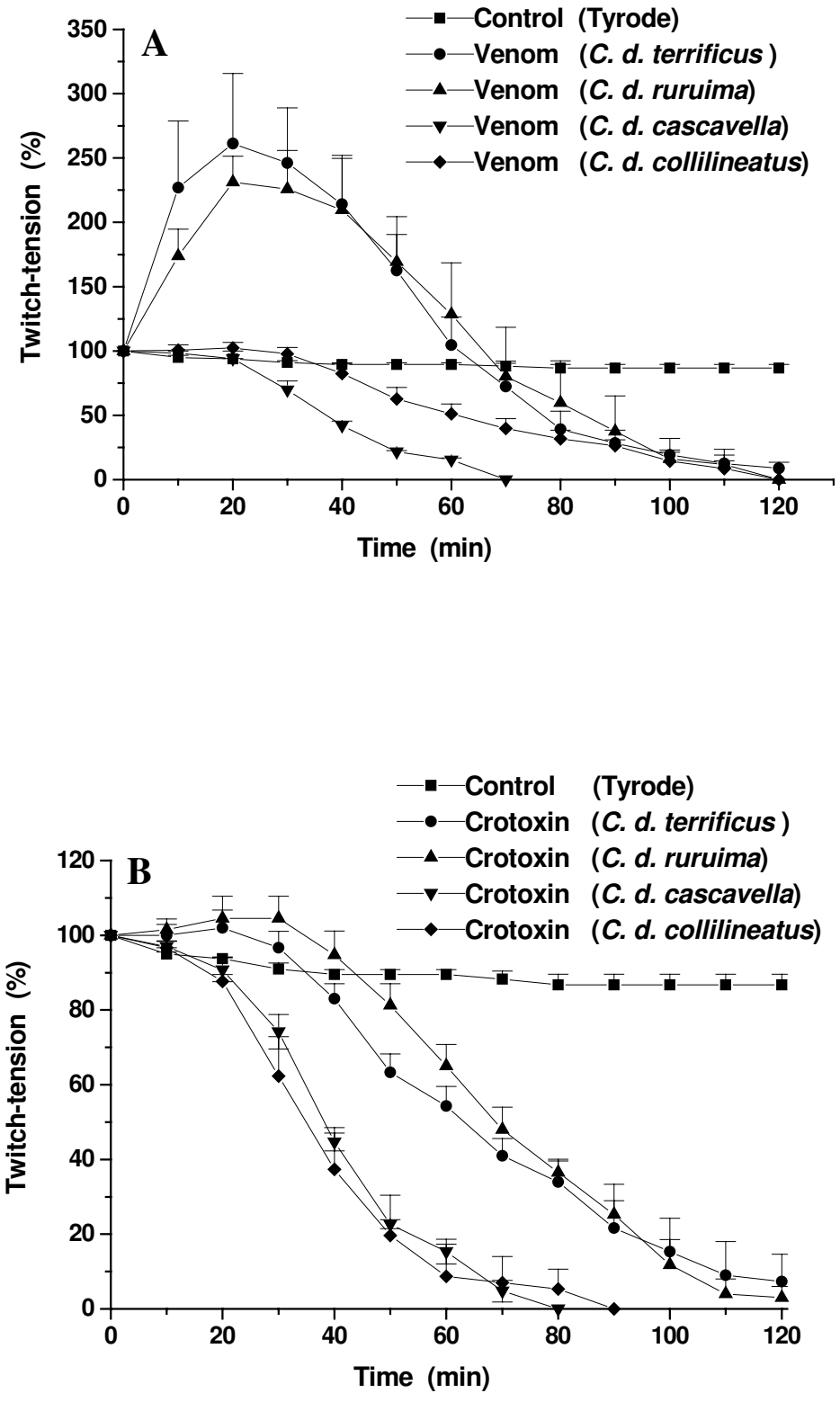
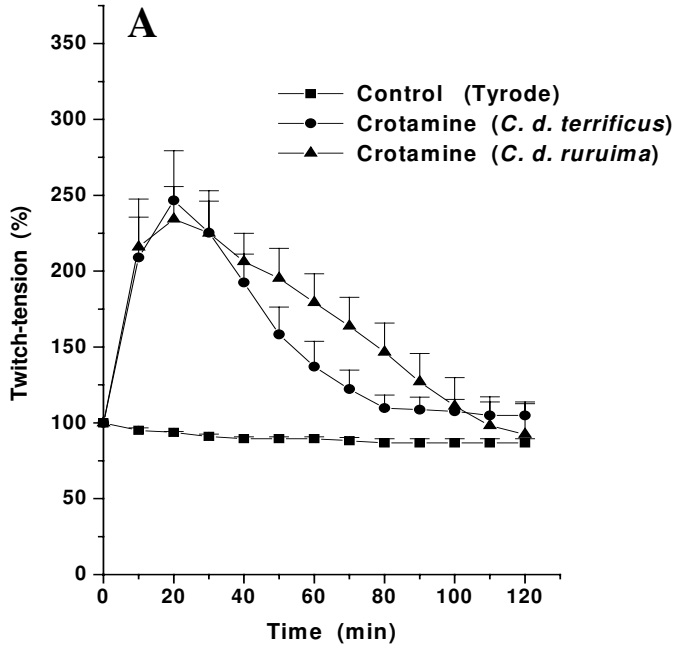
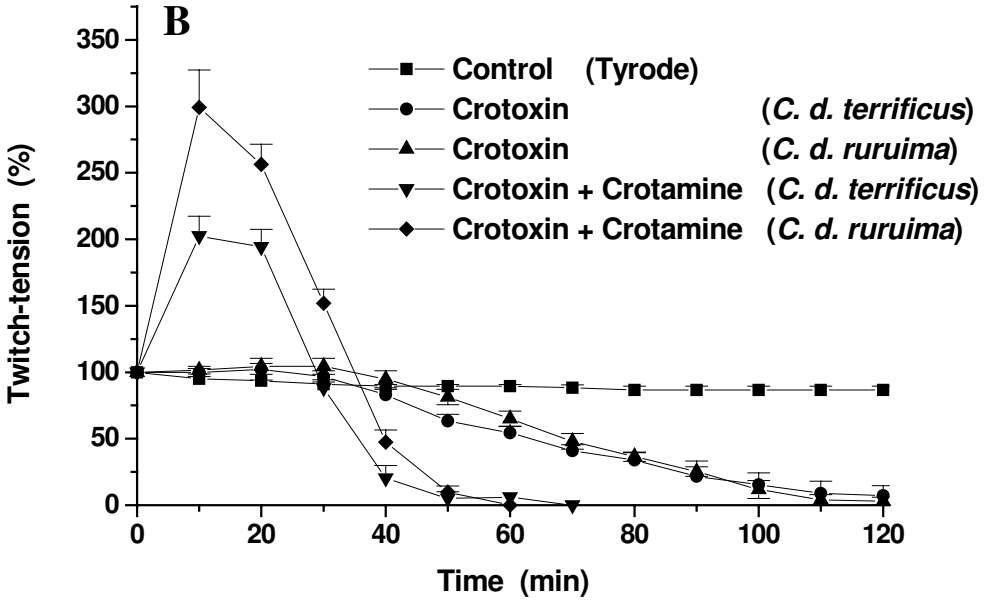


Fig. 2. Hernandez-Oliveira et al. – Comparison of the neurotoxic effects of *Crotalus durissus terrificus*.....





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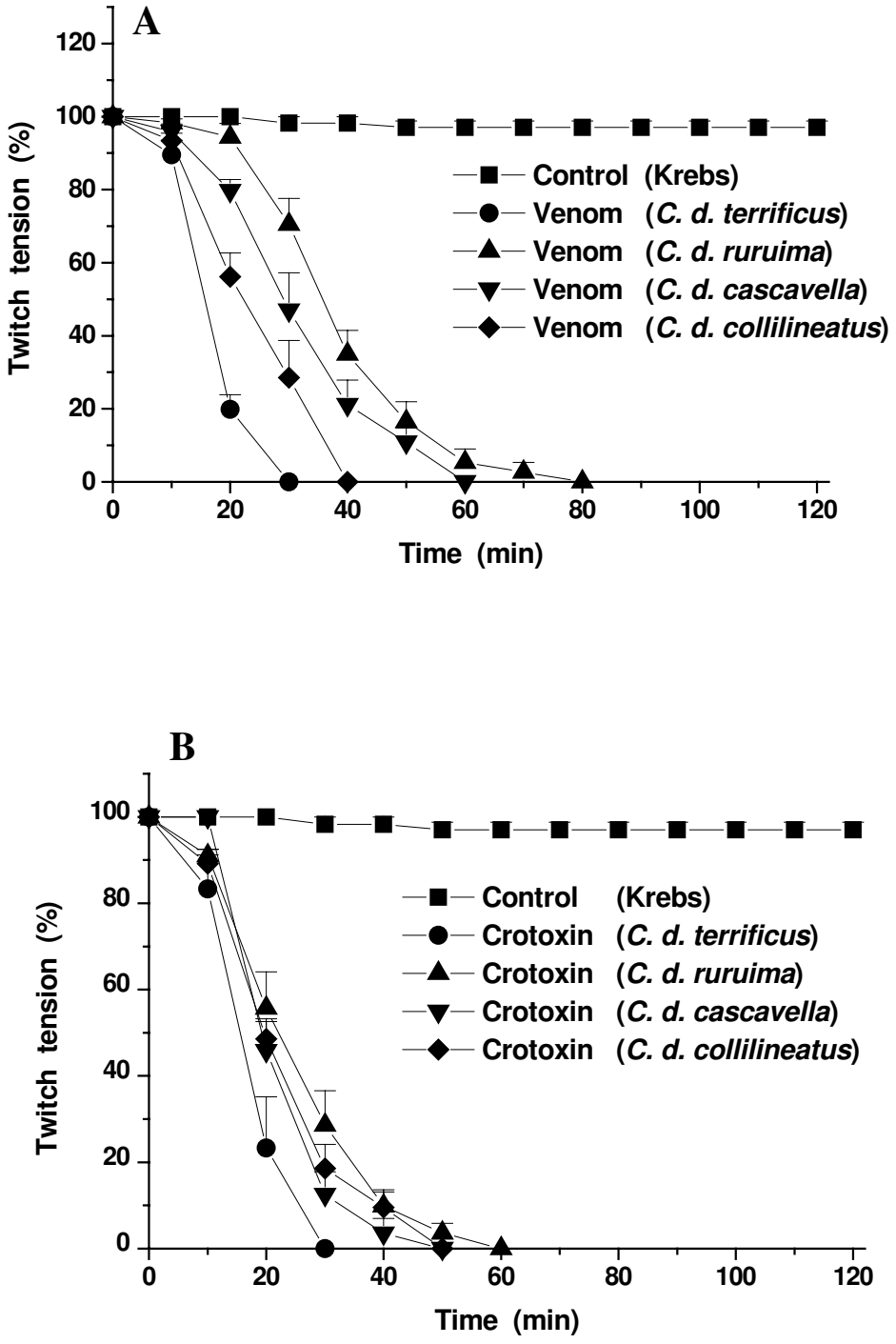

Fig. 4. Hernandez-Oliveira et al. – Comparison of the neurotoxic effects of *Crotalus durissus terrificus*.....

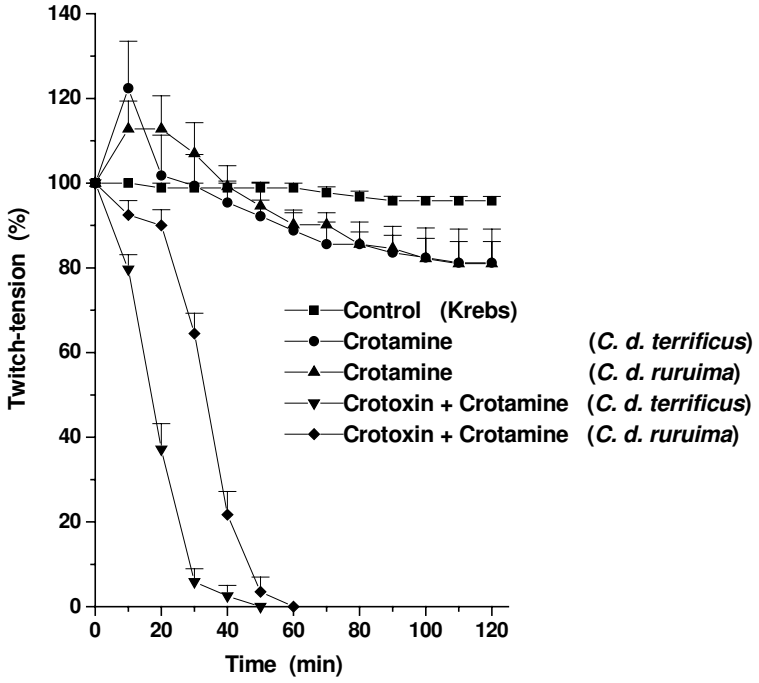

Fig. 5. Hernandez-Oliveira et al. – Comparison of the neurotoxic effects of *Crotalus durissus terrificus*.....

Comparative study between the crotoxin isoforms from *Crotalus durissus terrificus, C. d. cascavella* and *C. d. collilineatus* on two neuromuscular preparations

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Running title: The crotoxin isoforms from *Crotalus* genus

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Abstract

In Brazil, the Crotalus durissus terrificus subspecie is the most studied, particularly concerning its crotoxin. Crotoxin is the major toxic component of the South American rattlesnake Crotalus durissus venom. It is composed of two different subunits, CA called crotapotin and CB weakly toxic phospholipase A_2 (PLA_2) with high enzymatic activity. In this paper, we decided to make a study of the main toxic characteristics of crotoxin (CrTX) and CB fraction from the other subspecies, we have compared the neurotoxic effects of *Crotalus durissus cascavella* (cdc), *C. d. collilineatus* (cdcoll), and *C. d. terrificus* (cdt) PLA_2 and crotapotin isoforms, in mouse phrenic nerve-diaphragm and chick Biventer cervicis preparations.

In our previous studies, crotoxins from cdt, cdc and cdcoll venoms were fractionated as already described by elsewhere. All subfractions were assayed under myographic parameter resulting in the following selection F16 and F7 represent the subunits from cdt, crotapotin and PLA_2, respectively. The subfractions F3 and F4 are the crotapotin subunits, whereas the subfraction F6 is the PLA_2 from both cdc and cdcoll venoms. Together, these subfractions represents the native crotoxin and cause a neuromuscular blockade; however, alone they did not alter the basal responses on mouse phrenic nerve-diaphragm.

Throughout these experiments, a single concentration (20 µg/mL) of cdt, cdc and cdcoll fractions was used with phrenic nerve-diaphragm indirectly stimulated. As these venoms were more sensitive on BC than PND preparations, a minor, but also single concentration (10 µg/mL) was utilized.

The effects of crotapotin subunit from cdt (F7), cdc and cdcoll (F3,F4) on PND (A) and BC (B) preparations, crotapotin alone did not alter the basal response in both preparations.

The PLA_2 ubunit from cdt (F16), cdc and cdcoll (F6) on PND (A) and BC (B) preparations, differently from PND preparation, these phospholipases induced a intense paralysis of twitch tension ($p<0.05$ compared to control).

1. Introduction

The brazilian venomous snakes belong to Elapidae and Viperidae families, which the latter has the Crotalinae subfamily that possess the *Crotalus*, *Lackesis* and *Bothrops* genera. The subspecies from *Crotalus* genus in South American are represented principally by *Crotalus durissus terrificus*, *Crotalus durissus ruruima*, *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* (Santoro *et al*., 1999).

The main toxins presents in crotalic venom are gyroxin (Barrio, 1961; Alexander *et al*., 1988), convulxin (Prado-Franceschi and Vital Brazil, 1981), crotoxin (Slotta and Fraenkel- Conrat, 1938) and crotamine (Moura Gonçalves, 1950). The crotoxin is the main toxic constituent responsible by high toxicity of the venom (Vital Brazil, 1966; 1972), with molecular weight of 30 kDa. It is a complex containing two subunits, a basic component (phospholipase A_2, phosphatidate 2-acylhydrolase, EC 3.1.1.4) (Hendon and Fraenkel-Conrat, 1971; Breithaupt and Habermann, 1971; Breithaupt, 1976) and another acidic component crotapotin (Habermann and Breithaupt, 1978), with no enzymatic activity. Crotapotin is a chaperon protein because it works like a

carrier for the phospholipase A_2 (PLA_2), in native crotoxin complex, increasing the neurotoxic effect of PLA_2 (Rubsamen *et al*., 1971; Bouchier, 1991).

Snake venom PLA_2 exhibit a wide variety of pharmacological and physiopathological effects. In addition to their role in the digestion of the prey, they can be neurotoxic, myotoxic, and also able to interfere with coagulation processes (Dennis, 1997).

On the other hand, several snake venom PLA_2s evolved to become potent neurotoxins, developing selectivity for neuronal structures and recognizing specific protein acceptors. Neurotoxic PLA_2s from snake venoms, also called neurotoxins, cause death by respiratory failure. They act on the neuromuscular junction, primarily at a presynaptic level, inhibiting the release of neurotransmitter acetylcholine (Dennis, 1997).

In this work, we studied the pharmacological effects of crotoxin subunits from *Crotalus durissus cascavella* (cdc) and *Crotalus durissus colillineatus* (cdcoll) venoms and compared to that related to *Crotalus durissus terrificus*, in mouse phrenic nerve-diaphragm and chick biventer cervicis preparations, Crotapotin and new phospholipase A_2 isoforms isolated from the cdc and cdcoll crotoxin complex were recombinated between itself, and reproduced the same effects to that described to native crotoxin from cdt venom.

2. Materials and methods

2.1. Venoms and toxins

C. d. terrificus (cdt), *C. d. cascavella* (cdc) and *C. d. collilineatus* (cdcoll) venoms were donated by Prof. Dr. Sergio Marangoni, Biology Institute, Laboratory of Biochemistry, State University of Campinas (UNICAMP). Crotoxin was purified from these venoms as described by elsewhere (Beghini *et al*., 2000; Toyama *et al*., 2000; Ponce-Soto *et al*., 2002).

2.2. Animals

Male Swiss white mice (26-32 g) were supplied by the Animal Services Unit of UNICAMP. The mice were housed at 25°C on a 12 h light/dark cycle and had free access to food and water. Male chick (4-8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water . Male New Zealand white rabbits (2-3 kg) were purchased from established breeder (Granja de Coelhos Grota Azul, Paulínia, SP) and housed individually with free access to food and water. This project (protocol number 517-1) was approved by the institutional Committee for Ethics in Animal Experimentation (UNICAMP) and was done within the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.3. Mouse phrenic nerve-diaphragm preparation

The phrenic nerve and diaphragm were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsanguination. The nerve-muscle preparation was mounted as described by Bülbring (1946). Hemidiaphragms and phrenic nerves were mounted in 5 mL tissue baths containing Tyrode solution (pH 7.4, 37ºC) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1. The preparations were mounted on an electrode that enabled indirect (supramaximal voltage, 0.1 Hz, 0.2 ms) with impulses delivered from a Grass S4 stimulator (Grass Instruments, Quincy, MA, USA). The resulting muscle tension was recorded using a force displacement-transducer (BG 25 GM, Kulite Semiconductor Products, Inc., Leonia, NJ, USA) coupled to a Gould RS 3400 recorder (Gould Inc., Cleveland, OH, USA). The preparation was allowed to stabilize for at least 15 min before the addition of fractions crotapotin (CrTP 20 µg/mL), PLA_2 (20 µg/mL) and combinated CrTP + PLA_2 (10 µg/mL each).

2.4. Chick biventer cervicis (BC) preparation

Male chicks (4-8 days old) were killed with ether and the biventer cervicis muscle was removed (Ginsborg and Warriner, 1960) and mounted under a resting tension of 1 g in a 5 mL organ bath containing aerated (95%O_2 - 5%CO_2) Krebs solution (pH 7.5, 37^{o}C) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, NaHCO 25.0 and glucose 11.65. A bipolar platinum ring electrode was placed around the tendon and indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz,

0.2 ms). Muscle contractions and contractures were recorded with a BG-10 GM Kulite displacement transducer coupled to a Gould RS 3400 recorder. Contractures to exogenously applied acetylcholine (ACh, 110 mM for 60 s) and KCl (20 mM for 120 s) were obtained in the absence of nerve stimulation prior to the addition of toxin and at the end of the experiment. The preparation was allowed to stabilize for at least 15 min before the addition of CrTP (10 μg/mL) or PLA_2 homolog fractions (10 μg/mL) or recombination of CrTP plus PLA_2 (5 μg/ml, each). These substances were more sensitive in this preparation than PND preparation, reason by which this concentration was choice.

2.5. Statistical analysis

Each experiment was repeated at least three times. The results were expressed as the mean $\pm$ S.E.M., as appropriate. Student's t-test was used for statistical analysis of the data. Values of $p<0.05$ were considered significant.

3. Results

3.1. Subfractions selection

In our previous studies, crotoxins from cdt, cdc and cdcoll venoms were fractionated as already described by elsewhere (see Material and methods). All subfractions were assayed under myographic parameter resulting in the following selection (Table 1): F16 and F7 represent the subunits from cdt, crotapotin and PLA_2, respectively. The subfractions F3 and F4 are the crotapotin subunits, whereas the subfraction F6 is the PLA_2 from both cdc and cdcoll venoms. Together, these subfractions represents the native crotoxin and cause a neuromuscular blockade; however, alone they did not alter the basal responses on mouse phrenic nerve-diaphragm (see Discussion).

3.2. Effects of isolated subunits from crotoxin on mouse phrenic nerve-diaphragm (PND) and biventer cervicis (BC) preparations

Throughout these experiments, a single concentration (20 µg/mL) of cdt, cdc and cdcoll fractions was used with phrenic nerve-diaphragm indirectly stimulated. As these venoms were more sensitive on BC than PND preparations, a minor, but also single concentration (10 µg/mL) was utilized.

Figure 1 shows the effects of crotapotin subunit from cdt (F7), cdc and cdcoll (F3,F4) on PND (A) and BC (B) preparations, Note that crotapotin alone did not alter the basal response in both preparations (n=5-7 experiments).

The same protocol was used as shown in Figure 2 with PLA_2 subunit from cdt (F16), cdc and cdcoll (F6) on PND (A) and BC (B) preparations. Note that differently from PND preparation, these phospholipases induced a intense paralysis of twitch tension ($p<0.05$ compared to control). The time for inducing a 50% paralysis was 31±3 min (n=4), 36±3 min (n=4) and 49±8 min (n=4), respectively for cdt (F16) and cdc and cdcoll (F6).

3.3. Effects of the subunits recombination on phrenic nerve-diaphragm (PND) and biventer cervicis (BC) preparations

As already mentioned, isolated subunits from crotoxin did not alter the basal response on mouse PND preparation. However, combining crotapotin+PLA_2, in a 1:1 ratio, resulted in the characteristic blockade of contractile responses, when the mixture [F7+F16 (cdt, n=4), F3+F6 (cdc and cdcoll, n=3 for both) or F4+F6 (cdc and cdcoll, n=3 for both)] was exposed to PND (Fig. 3A) preparation ($p<0.05$ compared to control). The time (in min) required for blocking 50% of twitch tension is showed in Table 2.

Similar response was obtained when the same crotapotin+PLA_2 mixture [F7+F16 (cdt, n=4), F3+F6 (cdc, n=4 and cdcoll, n=5) or F4+F6(cdc and cdcoll, n=4 for both)], in a 1:1 ratio, when exposed to BC preparations (Fig. 3B, $p<0.05$ compared to control), but showing clearly the major sensitivity of these subunits reconbinated on chick preparation (Table 2).

3.4. Pharmacological responses face to exogenous ACh and KCl addition on BC preparations

The responses to exogenous ACh or KCl addition on BC preparations exposed to experiments above were unaffected after the complete blockade of the twitch-tension response (Table 3).

4. Discussion

The methodology used to purify crotoxin and their constituents, PLA_2 and CrTP, from cdt, cdc and cdcoll venoms, revealed different isoforms in *Crotalus* genus (Toyama *et al*., 2000; 2003; Beghini *et al*., 2000; Ponce-Soto *et al.,* 2002). Our results demonstrated that the CrTP and PLA_2 association represents the native CrTX separate in its two components, no biological effects on the neuromuscular in phrenic nerve-diaphragm preparation. Assayed isolatly they have no biological effects at mouse neuromuscular junction, as already described by elsewhere (Slotta and Fraenkel-Conrat, 1938; Chang and Lee, 1977; Hawgood and Smith, 1977; Hawgood and Santana de Sá, 1979; Bon 1982).

It is well known that the activity of crotoxin depends on a molecular synergism in which the non-toxic, acidic subunit crotapotin potentiates the toxicity of the enzymatically active basic PLA_2 (Habermann and Breithaupt, 1978; Bon *et al*., 1989). By the first time, a result totally different was found to PLA_2, which alone (F16 cdt, F6 cdc and cdcoll,) presented blocking effect on chick biventer cervicis preparations. In this case, crotapotin was not essential for discharging the enzymatic activity of PLA_2 and subsequent neuromuscular action on this preparation, an unexpected and unpublished result.

The enzymatic characteristics of this PLA_2s (F16 cdt, F6 cdc and cdcoll) were similar to those of other PLA_2s, such as F16 (Toyama *et al.,* 2000, Hernandez-Oliveira *et al*., 2005 (accepted), F15 (Toyama *et al.,* 2003) and F17 (Oliveira *et al.,* 2002) from the venom of *C. d. terrificus* and also to Cdcolli F6 from the venom of *C. d.*

collilineatus (Ponce-Soto *et al.,* 2002) and Cdcasca F6 from the venom of *C. d. cascavella* (Beghini *et al.,* 2000).

There are PLA_2 with no enzymatic activity as that found in bothropic venom such as bothropstoxin-I, from *B. jararacussu* venom (Heluany *et al*., 1992; Soares *et al,*. 2000a; b; 2001; 2002; Oshima-Franco *et al*., 2004) and *B. insularis* (Cogo *et al*., 1998 Lobo de Araújo *et al*., 2002), which have been shown to produce neuromuscular blockade.

The PLA_2 related here (cdt, cdc, cdcoll) presented similarity in producing neuromuscular blockade on chick biventer cervicis preparations, maybe it is because of residues of amino acids in the region C-terminal are similar to the found in the bothropic venom. The presence of amino acids in the region C-terminal can be that explains the neurotoxicity of this PLA_2 in the neuromuscular blockade on chick biventer cervicis preparation. The importance of this region C-terminal and the presence of residues of basic amino acids has been established for so many authors (Gutiérrez and Lomonte, 1995; Selistre de Araujo *et al.,* 1996; Gutiérrez and Ownby, 2003; Lomonte *et al.,* 2003).

In contrast, in mouse phrenic nerve-diaphragm preparations, the neuromuscular blockade produced by the same concentration of toxin was dependent of crotapotin.

The *in vitro* CrTP (cdt F 7; cdc and cdcoll F3 and F4) plus PLA_2 (cdt F16, cdc and cdcoll F6) recombination was capable to reproduce the same blocker effect related to CrTX on isolated neuromuscular preparation. This neurotoxic effect was explained as the following: CrTP induces PLA_2 to a new conformational state exploiting pharmacological sites and inactivating catalytic sites; this way, the crotoxin complex

can act with the receptors from target cells (Yang, 1994, Gutiérrez and Lomonte, 1995, Kini, 1997).

These present results confirm the integrity of crotoxin complex in their molecular rearrangement to producing the neurotoxic effect but also show that this optimized methodology conserved the biological function from isolated proteins.

The high level of purity from isolated fractions show that the crotoxin complex recombination represents is due to an equilibrium of charges between CrTP and PLA_2 for reproducing the neurotoxic effect. Faure and Bon (1988) detected 8 crotoxin isoforms from cdt venom, as result of exogenous expression responsible by two amino acids change up to the report of several positions in the PLA_2 sequence. Faure (1994) showed that the multiplicity of isoforms from cdt CrTX is the result of post-transdutional change a part from a precursor.

The *in vitro* CrTP+PLA_2 recombinated showed a potent neurotoxic activity on mouse phrenic nerve-diaphragm. All of the preparations induced the neuromuscular blockade, except CrTP and PLA_2 (F4+F6) cdc, that apresented 80 % blockade, but the *in vitro* F3+F6 cdcoll recombination was the most potent, blocking totally at 70 min.

The characterisc facilitatory observed before the neuromuscular blockade any was seen only in these preparations as much PND as BC.

The isolation and biochemical characterization of these enzymes has great importance and the catalytic activity study becomes possible the pharmacological correlation. Neurotoxins are important tools in the ionic channels investigation and neurotransmission studies and can contribute to the new methodology development as

well as in the determination of a structural molecular model of sodium channels (Smithies, 1979; Vassilev *et al*., 1988).

In conclusion, F16 and F6 PLA_2 isoforms purified from the venom of *C. d. terrificus*, *C. d. cascavella and C. d. collilineatus*, exerts neurotoxicity that partly involves the residues of basic amino acids but is apparently independent of the presence of crotapotin only observed in BC preparations. Also the crotapotin isoformas and PLA_2 of these venoms presented similar effects in the same preparation.

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Effects Commercial Crotalic antivenom and produced in rabbit antiserum neutralize the effects neurotoxicity of *Crotalus durissus ruruima* venom crotoxin and PLA_2 its in chick *Biventer cervicis* preparations.

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Key words: *Crotalus durissus ruruima*; *C.d. collilineatus*; crude venoms; crotoxin; anti-serum; neutralization; neuromuscular blockade; phosphalipase $A_{2;}$ chik *Biventer cervicis*

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Abstract

The high mortality caused by *Crotalus durissus ruruima* snake venom is mainly due to crotoxin, which acts on the neuromuscular junction inhibiting the mechanism mediating acetylcholine release, thus leading to motor and respiratory paralysis and subsequently to animal death.The crotoxin molecule is composed of two subunits, an acidic non-toxic and non-enzymatic polypeptide named crotapotin and a weakly toxic basic phospholipase A_2 (PLA_2).

In this work, we examined the ability of rabbit anti-sera against crude venom, crotoxin from *C.d. ruruima* and its PLA_2 from *C.d. collilineatus* subunit to neutralize the neurotoxicity of crude venom and crotoxin from *C. d. ruruima* in chick Biventer cervicis preparations.

Antibody titer and the specificity of the antivenoms produced were evaluated by ELISA. This was confirmed by ELISA, with anti-sera having end-point dilutions of 3 x 10 (-6).

Anti-venom and anti-crotoxin from *C.d. ruruima* and anti-PLA_2 from *C.d.collilineatus* serum neutralized the neuromuscular blockade in chick Biventer cervicis preparations at venom or crotoxin: anti-serum ratios of 1:1, respectively. Anti-PLA_2 serum also neutralized this neuromuscular activity at a venom or crotoxin:anti-serum ratio of 1:1.

The neutralizing capacity of the sera in chick biventer cervicis preparations was comparable to that of commercial anti-serum raised against *C. d. ruruima* venom. These results show that anti-sera against venom and crotoxin from *C. d. ruruima* and PLA_2 from *C. d. collilineatus* neutralized the neuromuscular blockade induced by venom and crotoxin in chick preparations, with the anti-serum against crotoxin being slightly less potent than that against crotoxin.

So much the venom, as the studied crotoxin induced the release of CK significantly different from the control. However, the inhibition of the preparation to the anti-venoms didn't reduce the high levels of CK significantly after 90 incubation min as it was waited. That result revelated that possibly the amount of proteins of the antivenoms interferes in the mechanism liberation of CK, suggesting that the enzymatic determination can constitute a quantitative parameter of mionecroses. It can be ended that, the study of the neutralization with antivenins produced of purified fractions, associate to the parameters miografic and biochemical, they can constitute an useful model in the elucidation of the mechanism of the liberation of CK in the neurotoxicity and miotoxicity of those toxins.

1. Introduction

In Brazil, the crotalic serpents are represented by just one species, *Crotalus durissus*. The most studied subspecies are *Crotalus durissus terrificus; C.d. ruruima; C.d. cascavella; C.d. collilineatus* and *C.d. marajoensis.*

Venom from subspecies *C.d.terrificus* is well characterized and represents the crotalic envenomation from any of the subspecies catalogued in the country.

Crotalic envenomation is characterized by systemic manifestations originated from neurotoxic, miotoxic and coagulating venom activities (Azevedo-Marques *et al*., 2003).

Crotoxin is a 23-kDa protein, isolated from *C.d. terrificus* venom (Slotta & Faenkel-Conrat, 1938), represents 60 to 75% of the total dry weight of the venom and constitute the major determining factor of its toxicity (Nascimento *et al*., 1996a; Vital Brazil, 1966). It presents a strong neurotoxic activity, produzing blocking of the neuromuscular transmission and death due to respiratory paralysis in laboratory animals (Vital Brazil, *et al.,* 1966; Breithaupt *et al*., 1974). Its action is predominantly pre-synaptic, inhibiting the release of acetilcholine by the motors nervous terminations (Chang & Lee,

1977; Hawgood & Smith, 1977). Besides the neurotoxic action, crotoxin has a miotoxic (Azevedo-Marques *et al*., 1985), hemolytic (Rosenfeld, 1971) and platelet aggregation activity (Landucci *et al.,* 1994).

Crotoxin is constituted of a basic subunit with PLA_2 activity and by an acid subunit non-enzymatic, named crotapotin (Hendon & Fraenkel-Conrat, 1971; Breithaupt e*t al*., 1974; Breithaupt, 1976). Both subunits acts in synergism, however isolated, PLA_2 is not much toxic and crotapotin is non-toxic (Rubsamen e*t al.,* 1971; Bon e*t. al*., 1979).

Since the beginning of the century, antivenom administration has been the most efficacious treatment for envenomation by *C.d.terrificus* (Brazil, 1903). Commercial anti-*C. d. terrificus* antivenom is raised in horses and may be either monovalent (*C. d. terrificus* venom only) or polyvalent (*C. d. terrificus* + *Bothrops* spp venoms). The theree principal producers of anti-*C. d. terrificus* antivenoms in Brazil are the Instituto Butantan (São Paulo, SP), the Fundação Ezequiel Dias (Belo Horizonte, MG) and the Instituto Vital Brazil (Rio de Janeiro, RJ).

Although antivenom therapy is effective in reducing the mortality associated with envenomation by *C.d.terrificus* (Ministério da Saude, 2001; Bochner, et al., 2003), little is known of the capacity of commercial antivenoms to neutralize the activities of the different components of *C. d. terrificus* venom, particulary crotoxin (Barral-Netto and von Sohsten, 1991; Barbosa et al., 1995).

The complex composition of snak venom, its specificity and the existence of serpent subspecies, determinate the needs to know its antigenic relations to the imunologic systems of animals that produce serum, bearing mind that heterologic serum consist of the most reliable treatment to the envenomation provoked by reptiles.

In the present study, we examined the ability of anti-venom raised in rabbits against crude venom (anti-venom), crotoxin (anti-crotoxin) from the *C. d. ruruima* and

PLA_2 (anti-PLA_2) from the *C. d. collilineatus,* venom to neutralize the neurotoxicity of this venom and its crotoxin. Neurotoxicity was assessedd by the ability venom and crotoxin to cause neuromuscular blockade in electrically stimulated chick *Biventer cervicis* preparations. Neutralization was tested by preincubating that anti-sera with venom or crotoxin and then assaying the residual biological activity.

2. Material and Methods

2.1. Venoms and reagents

C. d. ruruima (cdr), venom were provided by the Institute biology UNICAMP, laboratory Prof Dr Sergio Marangoni. Solvents (HPLC grade) and other reagents were acquired from Sigma and Aldrich Chemical Company (St Louis, MO, USA).

2.2. Animals

Male chick (4-8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water . Male New Zealand white rabbits (2-3 kg) were purchased from established breeder (Granja de Coelhos Grota Azul, Paulínia, SP) and housed individually with free access to food and water. The experiments described here were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA)

2.3. Anti-venom

The commercial crotalic anti-venom used was a semi-purified immunoglobulin-rich solution produced by hyperimmunization of horses with *C. d. terrificus* venom

(Instituto Vital Brazil, Rio de Janeiro, RJ, Brazil) This anti-venom is distributed for use after peptic digestion and partial purification by ammonium sulfate precipitations, and contains 0,35% phenol as preservative.

2.4. Anti-sera

Rabbits were immunized by successive i.m. inoculation with 800 μg of venom, crotoxin (CrTX) and PLA_2 per rabbit. In all of the injections were administered with Freund´s complet adjuvant (Difco Laboratories,Detroit, USA) in a 1:1 ratio. The apearence of antibodies in the serum of the rabbits was monitored biweekly by gel immunodiffusion (Ouchterlony, 1949) and was confirmed by ELISA (Chávez-Olórtegui et al., 1997) at the end of the experiment. Blood samples were collected from a marginal ear vein and stored at 4°C overnight. The sera were subsequently separated by centrifugation and aliquots were stored at - 70°C.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 wells) were coated overnight at 4°C with 100 μl of venom, crotoxin or PLA_2 (5 μg/well) in sodium bicarbonate buffer. The plates were washed three times with saline solution containing 0.5% tween 20 and unbound sites were blocked for 1 h at room temperature with 2% bovine casein in phosphate-buffered saline (PBS). The plates were again washed (three times) with saline solution containing 0.5% tween 20 and used immediately for ELISA. To measure the serum titers, 100 μl of serial dilutions of serum in PBS containing 0.25% bovine casein and 0.05% tween 20 were added to the plates and incubated for 1 h at room temperature. The plates were washed again and incubated for 1 h with 100 μl of a goat anti-rabbit immunoglobulin G (whole

molecule) – peroxidase conjugate (Sigma, St Louis, MO, USA; 1:1000 in PBS containing 0.25% bovine casein and 0.05% Tween 20), followed by further washing. The substrate solution for the peroxidase assay (citrate buffer, pH 5.0, containing 0.2mg of *o*-phenylenediamine/ml and 0.5 µl and 0.5 µl of 30% H_2O_2/ml) was added and the enzymatic reaction allowed to proceed for 15 min in the dark at room temperature. The reaction was stopped with 50 µl of 5% H_2SO_4 and the absorbance was read at 492 nm with a SpectraMax 340 multi-well plate reader (molecular Devices, Sunnyvale, CA, USA).

2.6. Purification of crotoxin (CrTX) homolog (Crotalus durissus ruruima) MolecularExclusion HPLC

Crotalus durissus ruruima venom and crotoxin homolog were purified as described else were Toyama et. al., 2000 and PLA_2 *C.d.collilineatus* described by Ponce-Soto et al., 2002.

The crotoxin purified from *C. d. ruruima* venom was obtained using the same method. Cdr (20 mg) was loaded onto a Protein-Pack 300 SW column (0.78 cm x 30 cm) and eluted with 0.25 M ammonium bicarbonate, pH 7.9, at a flow rate of 3.0 ml/min. The elution profile was monitored at 280 nm using a Waters UV/visible detector model 490 and fractions were collected using a Foxy 200 automatic fraction collector. The chromatographic runs were recorded using a Waters recorder model 746 data module-dual channel integrator. The fractions corresponding to crotoxin were lyophilized and stored at -20°C until further purification by reversed-phase HPLC.

2.7. Chick biventer cervicis (cBCP) preparation

Male chicks (4-8 days old) were killed with ether and the *biventer cervicis* muscle was removed (Ginsborg and Warriner, 1960) and mounted under a resting tension of 1 g in a 4 ml organ bath containing aerated (95%O_2 - 5%CO_2) Krebs solution (pH 7.5, 37^{o}C) of the following composition (in mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, NaHCO 25.0 and glucose 11.65. A bipolar platinum ring electrode was placed around the tendon and indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz, 0.2 ms, 3-4 V). Muscle contractions and contractures were recorded with a BG-10 GM Kulite displacement transducer coupled to a Gould RS 3400 recorder. After 20 min, contractures to exogenously applied acetylcholine (ACh, 110 μM for 60 s) and KCl (20 mM for 120 s) were obtained in the absence of nerve stimulation prior to the addition of toxin and at the end of the experiment, as a further test for the presence of neurotoxic and miotoxic activities (Harvey et al., 1994).

2.8. Creatine Kinase release (CK)

Eletrically stimulated biventer cervicis preparations were incubated with venom and crotoxin and anti-venom, anti-crotoxin, anti-PLA_2 and commercial crotalic anti-venom, as described above. At 0, l5, 30, 60, 90 min after venom addition, samples of the bath solution (100 μl) were collected and stored at 4°C until creatine kinase activity was measured using commercial kit (Granutest® 2.5, Diagnostica, Merch, Germany).

The samples were replaced with an equal volume of fresh solution. The enzyme activity was expressed as international units/liter (IU/l).

2.10. Statistical analysis

The results were expressed as the mean ± S.E.M, as appropriate. Student's t-test was used for statistical analysis of the data. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Antibody production

Antibody production during the immunization process was monitored by double immunodiffusion until strong immunoprecipitin bands were consistently obtained (results not show) at which point the rabbits were bled and the antibody titers of the sera determined by ELISA (Fig.02) shows the reactivity of crude venom, crotoxin *C.d. ruruima* and PLA_2 *C.d.collilineatus*.venom with serial dilutions of the anti-venom, anti-crotoxin and anti-PLA_2 sera. In all cases, reactivities were with the corresponding immunogen (crude venom, crotoxin, PLA_2 venom for anti-venom, anti-crotoxin and anti-PLA_2 sera, respectively). The endpoint dilutions of the anti-venom, anti-crotoxin anti-PLA_2 sera were similar (3×10^6).

3.2 Neuromuscular activity its neutralization

Neuromuscular activity was assayed single concentrations of *C.d. ruruima* venom or crotoxin in the chick Biventer cervices preparations (10 μg/ml each). These concentrations were chosen based on preliminary experiments in which they produced neuromuscular blockade within a reasonable timescale. The times required for 50% neuromuscular blockade were 35 ± 2 min and 24 ± 3 min for the crude venom and crotoxin, respectively (each at 10 μg/ml). Fig.01. (A and B).

3.2.1. Chick Biventer cervices preparations

Figure 01 (A) and (B) shows the neutralizing capacity of anti-venom, anti-crotoxin (*C.d.ruruima*) and anti-PLA_2 (*C.d.collilineatus*) sera against the neuromuscular blockade produced by *C.d. ruruima* venom. Anti-venom, anti crotoxin protected against the neuromuscular blockade at crude venom and crotoxin anti-venom and anti-crotoxin ratio ≥ 1:1 ($p < 0.05$, $t_{20 - 90\ min}$), while anti-PLA_2 *C. d. collilineatus* protected crude venom and crotoxin (ratio ≥ 1:1 ($P < 0.05$, $t_{20 - 90\ min}$).

The efficacy of commercial anti-venom examined in this preparations show effect protected against the neuromuscular blockade at crude venom and crotoxin, commercial anti-venom and ratio ≥ 1:1 ($P < 0.05$, $t_{20 - 90\ min}$).

3.3. Study of the Creatine Kinase release (CK)

The determination of the niveis of set free CK in the way of incubation using itself it concentration of 10 μg/ml of crude venom and crotoxin of *C.d. ruruima*, was gotten through aliquot removed of it eliminates gift in the bath. All the muscles chick biventer cervices preparations incubated with the different assayed venoms , had shown significant increase ($p < 0.05$) in the values of CK, relation to the value it has controlled from 20 minutes of incubation of the preparation. The crotoxin was the one that had greater release of this enzyme in relation with crude venom, for the chosen concentration. The CK release was diminished in significant way ($p< 0.05$) for the commercial anti-venom crotalic and anti-venom, anti-crotoxin and anti-PLA_2 (*C.d. collilineatus*), these had been efficient in neutralizing the induced release of CK for crude venoms and crotoxin in chick biventer cervicis preparations. Fig. 03

4. Discussion

The biological activities of 'yellow' and 'white' venom of a rattlesnake *Crotalus durissus ruruima* Hoge, 1965, found in the savanna-like vegetation (cerrado) of northern Brazil (Roraima) and Venezuela have been studied, and compared to the reference *Crotalus durissus terrificus* venom. The lethal activity of venoms depended on the inoculation route.

Dos Santos, et al., 1993, evidence the most toxic venom was the white one. The venoms of *C. d. terrificus* and the yellow of *C. d. ruruima* had similar lethalities. The yellow venom of *C. d. ruruima* a caseinolytic activity three times higher than that venom of *C. d. terrificus* or the white one of the *C. d. ruruima*. Hemorrhagic and necrotic activities were found only in the yellow venom.

White and yellow venoms from *C. d. ruruima* there is similar action on fibrinogen; this thrombin-like action was greater with *C. d. terrificus* venom. On histopathological sections local and pulmonary hemorrhage was found only with the yellow venom, but myonecrotic activity was observed with both venoms of *C. d. ruruima.*

The venom of this subspecies has a similar biochemical composition and biological activities to the more widely studied *C. d. terrificus* (Santoro et al., 1999).

While relatively well characterized biochemically, there has been no detailed immunological study of *C. d. terrificus, C. d. cascavella* and *C. d. collilineatus* venom, although Santoro et al., (1999) showed that the immunoreactivity of this venom with commercial crotalic anti-venom in ELISA.

As shown here ELISA confirmed the excellent reactivity of the anti-sera to crotoxin and PLA_2 with the highest being obtained with the corresponding antigen used in immunization. The quality of the rabbit anti-sera was also confirmed by their ability

totally protect against the neuromuscular blockade induced by *C. d. ruruima* venom and crotoxin in the nerve-muscle preparation tested. Commercial crotalic anti-venom also efficiently neutralized the blockade induced by venom or crotoxin in chick *biventer cervices* preparations. The slightly greater neutralizing capacity of this anti-venom compared the rabbit anti-venom may more apparent than real since commercial anti-venom are often semi purified, globulin-rich preparations, whereas rabbit anti-sera are generally used without further processing (Lomont et al., 1987).

In addition venoms are complex mixtures of different antigens and variety of potentially antigenic sites is presented to the immune system of the animal during immunization, in contrast to purified toxins, which are antigenically less diverse Althoug venoms may present more antigenic sites, note all of these are necessarily associated with the active site of toxins (Freitas et al., 1990).

As reported elsewhere (Freitas, et al., 1990; Oshima Franco et al., 1999, Beghini et al., 2004), antiserum produced against crotoxin from *C. d. terrificus and C. d. cascavella,* venom neutralized the principal lethal effects of this venom. Our results with anti-sera to crude venom, crotoxin from *C.d. ruruima*, and PLA_2 *C.d. collilineatus venom* extended these findings and showed very efficient neutralization of the neurotoxicity of the *C.d. ruruima* and PLA_2 *C.d.collilineatus* crude venom and crotoxin. The neutralization of the neuromuscular blocking activity of the venom by anti-crotoxin serum was expected since crotoxin is the principal toxin in the *Crotalus durissus* venom (Santoro et al., 1999; Beghini et al., 2000). These results support the observations of Freitas et al., (1990) that immunization with crotoxin and/or the inclusion of anti-crotoxin antibodies as a supplement in a polyclonal anti-venoms should be considered in order to obtain hyperimmuneserum with a high level of protection against the toxic effects of *C.d. terrificus* venom.

It became evident the release kinetics of CK to antivenom, the venom and the crotoxin. The increase in CK levels in some tests were significant, and it believed that it's due to the influence of antivenom also observed by Oshima-Franco et al., 1999, that tested antivenom only in EDL preparations and found Ck release significantly high in relation to the venom and crotoxin form crotoxina de *C.d.terrificus.* One possible explanation is could be the protein concentration in the solution.

Suarez-Kurtz, 1982, working with frog electric muscles, demonstrated that CK, even though it is an enzyme of considerable size (81 Kda), it can be released by osmotic alterations, with apparently no irreversible cellular damage, explaining our results when we used antivenom with venom and crotoxin.

Our findings for *C. d .ruruima* agree with these studies since the anti PLA_2 (c. d. collilineatus) serum protected against the neuromuscular blockade caused by venom or crotoxin, and was slightly more efficient than the anti-crotoxin serum. This protection by anti-PLA_2 further confirms the central role of PLA_2 in toxicity of crotoxin and supports works showing that inactivation of PLA_2 activity with p-bromophenacyl bromide or the removal of calcium ions abolished the neurotoxicity of crotoxin (Marlas & Bon 1982).

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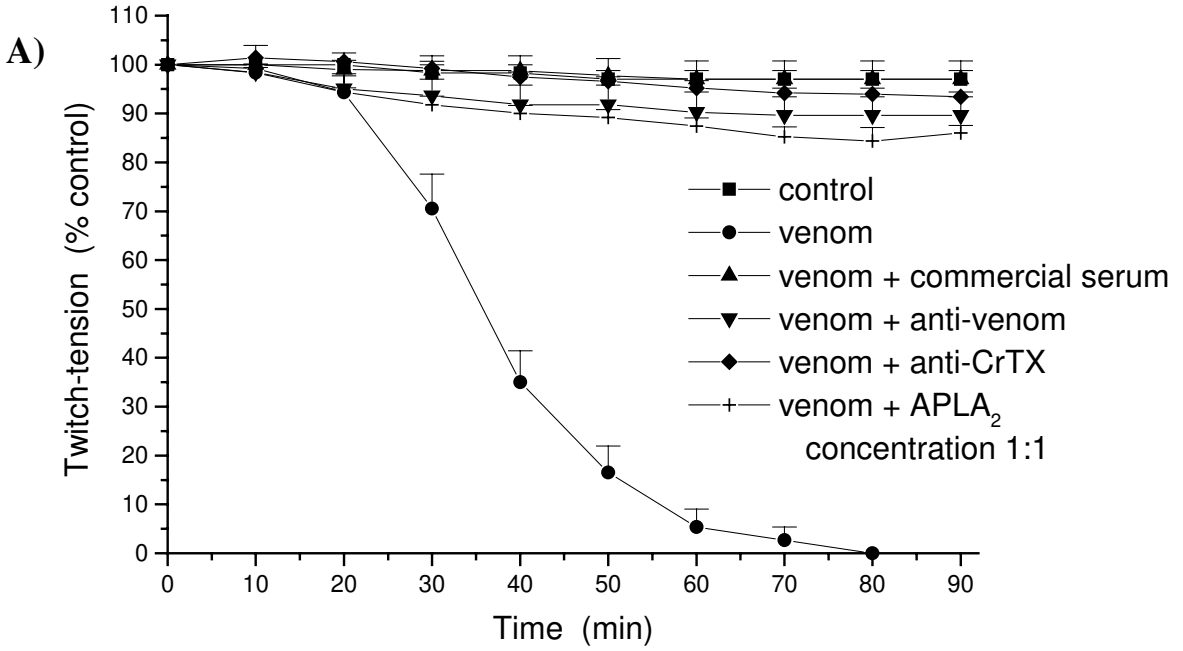
Legends

Figure 01. Neutralization by commercial anti-venom crotalic, anti–venom, anti-crotoxin specific *C.d. ruruima* and anti PLA_2 *C.d.collilineatus*, sera of the neuromuscular blockade by *C. d ruruimain* chick biventer cervices preparations. The crude venom and crotoxin alone caused significant neuromuscular blockade. Panels (A) and (B) show the protection by anti–venom, anti-crotoxin, anti- PLA_2 and commercial anti-venom crotalic, anti-sera against the neuromuscular blockade induced by *C.d. ruruima* venom and crotoxin respectively. Each point is the mean ± SEM of 6-7 experiments . In the anti-venom groups A and B, all time points from 20 min onwards were significantly different ($p < 0.05$) from venom or crotoxin alone.

Figure 02. ELISA reactivity of anti-venom (A), anti-crotoxin (B) *C.d. ruruima* and anti- PLA_2 (C) *C.d. collilineatu*s with venom and crotoxin from *C.d.ruruima* and PLA_2 from *C.d.collilineatus*. The plates were coated with antigen (5 μg/well) then incubated with anti-serum at the dilutions indicated and finally with an appropriate IgG - peroxidase conjugate and substrate (OPD). The resulting absorbances were read at 492 nm and each point is the mean ± SEM of four determinations.

Figure 03. Neutralizante effect of the commercial antiserum antivenom specify on the CK release induced for crude venom and crotoxin from *C.d. ruruima* after 90min of incubation. Each point represents the average ± EPM de 6-12 experiments, $p < 0.05$ in relation to the control. In A crude venom, B venom+antivenom; C venom+anticrotoxin, D venom+antiPLA_2 *(C.d.collilineatus),* E venom+commercial serum. Em F crotoxin, G crotoxin+antivenom, H crotoxin+anticrotoxin, I crotoxin+antiPLA_2 *(C.d.collilineatus*), J crotoxin+commercial serum.

FIG. 01


A)
Twitch-tension (% control)
Time (min)
control
venom
venom + commercial serum
venom + anti-venom
venom + anti-CrTX
venom + APLA2
concentration 1:1

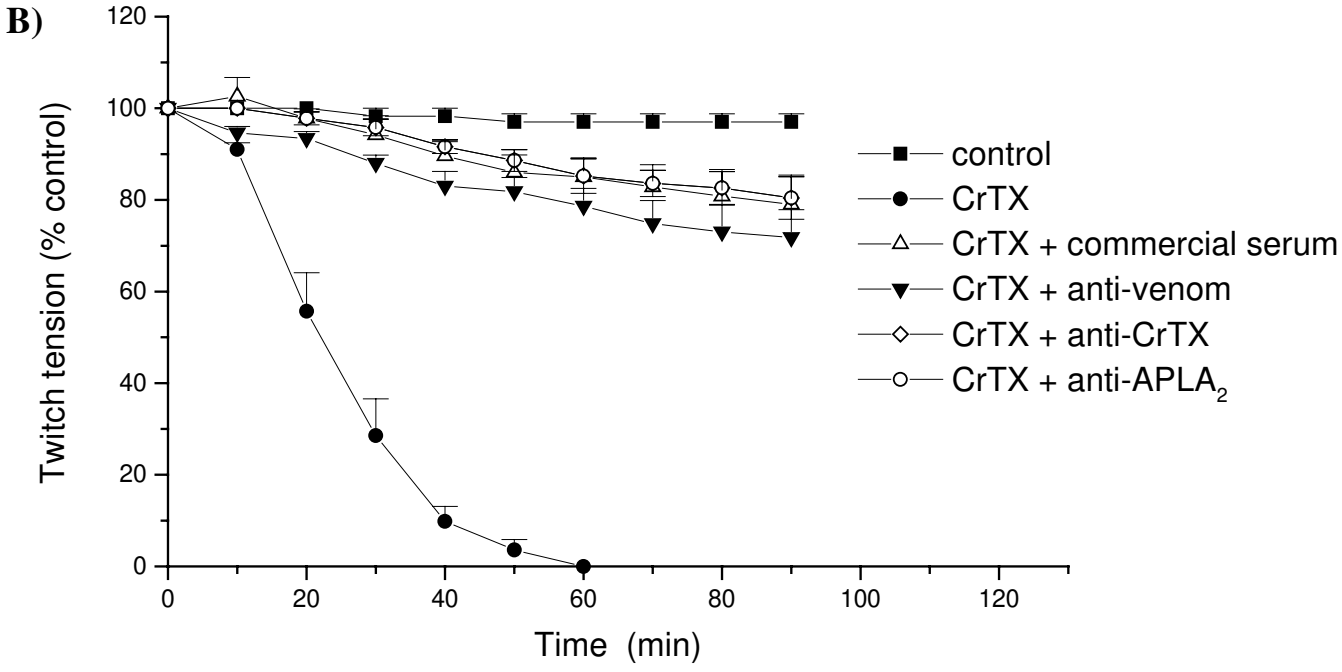
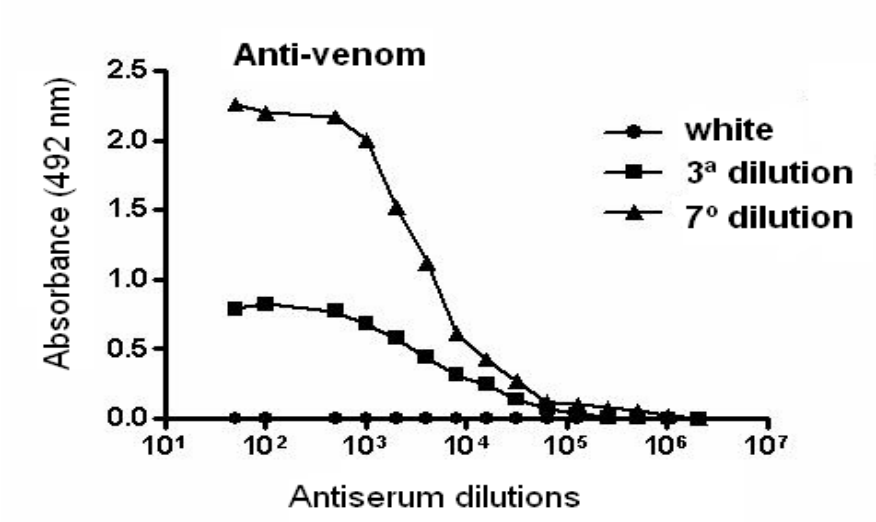
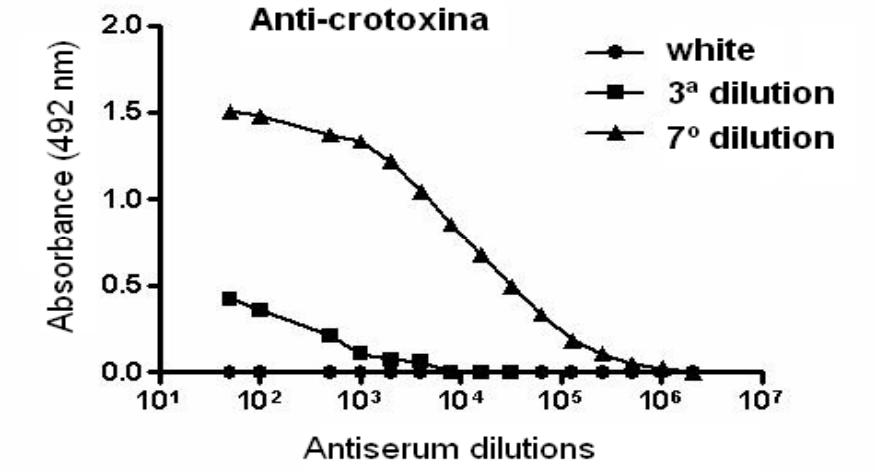

B)
Twitch tension (% control)
Time (min)
control
CrTX
CrTX + commercial serum
CrTX + anti-venom
CrTX + anti-CrTX
CrTX + anti-APLA2

FIG. 02

A)


Anti-venom
Absorbance (492 nm)
2.5
2.0
1.5
1.0
0.5
0.0
10^1 10^2 10^3 10^4 10^5 10^6 10^7
Antiserum dilutions
white
3ª dilution
7º dilution

B)


Anti-crotoxina
Absorbance (492 nm)
2.0
1.5
1.0
0.5
0.0
10^1 10^2 10^3 10^4 10^5 10^6 10^7
Antiserum dilutions
white
3ª dilution
7º dilution

C)

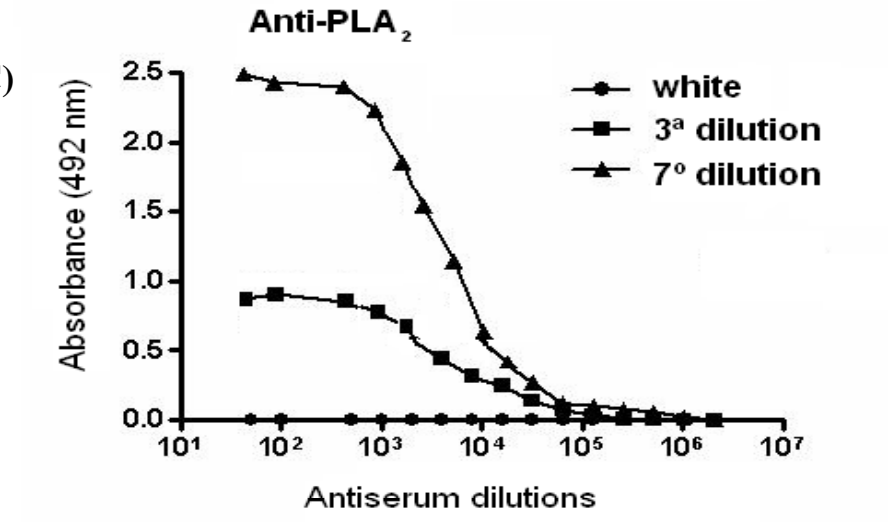
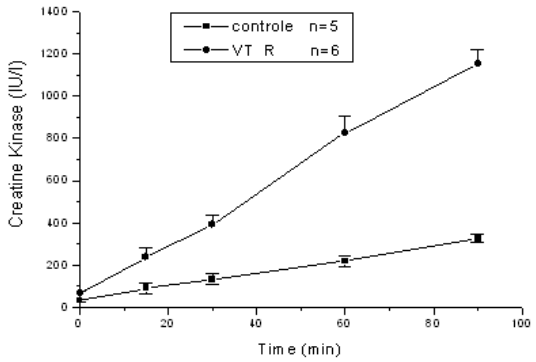
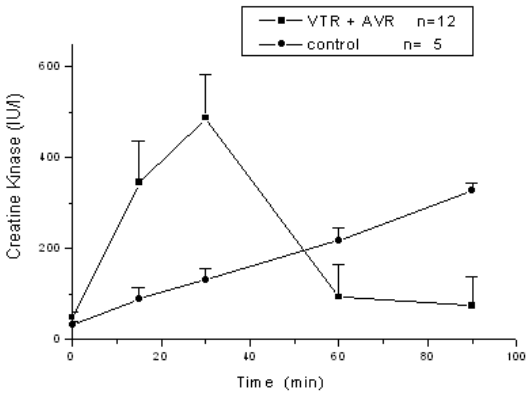

Anti-PLA$_2$
Absorbance (492 nm)
2.5
2.0
1.5
1.0
0.5
0.0
10^1 10^2 10^3 10^4 10^5 10^6 10^7
Antiserum dilutions
white
3ª dilution
7º dilution

FIG. 03

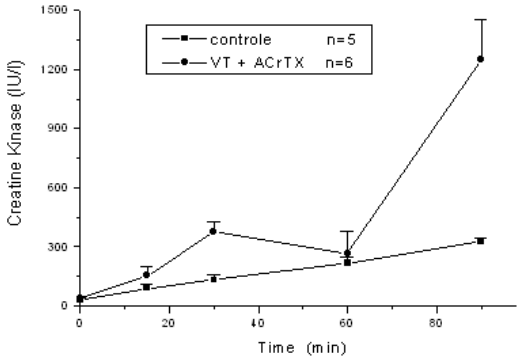
A)


controle n=5
VT R n=6
Creatine Kinase (IU/l)
Time (min)

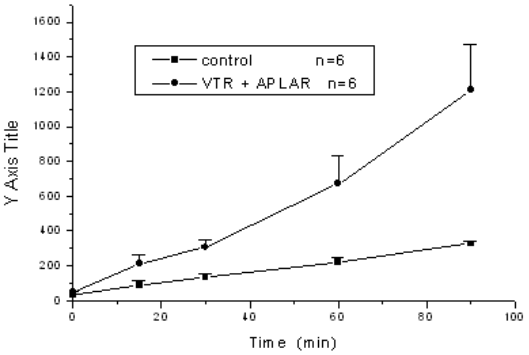
B)


VTR + AVR n=12
control n= 5
Creatine Kinase (IU/l)
Time (min)

C)


controle n=5
VT + ACrTX n=6
Creatine Kinase (IU/l)
Time (min)

D)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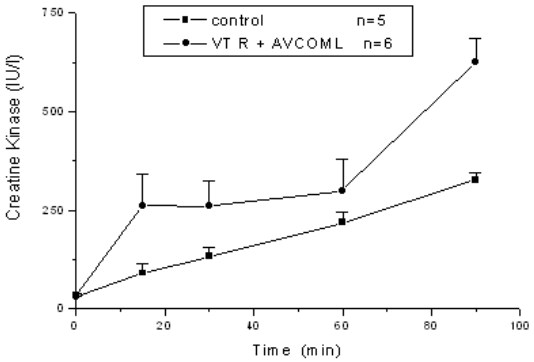
Y Axis Title
Time (min)

E)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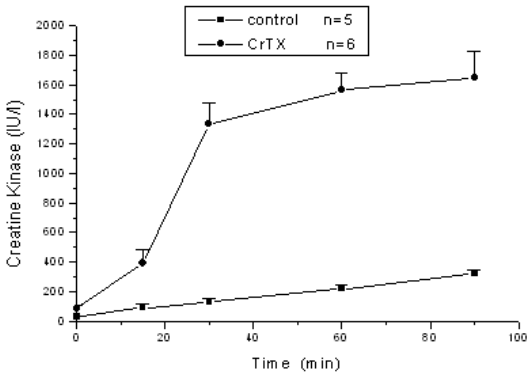
Time (min)

F)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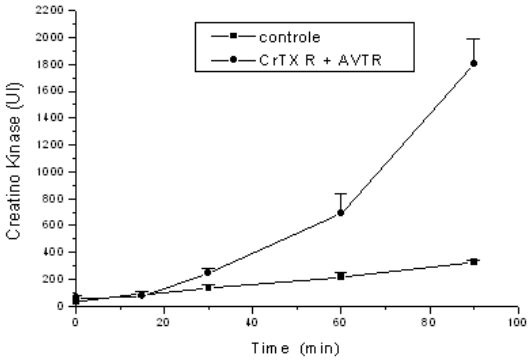
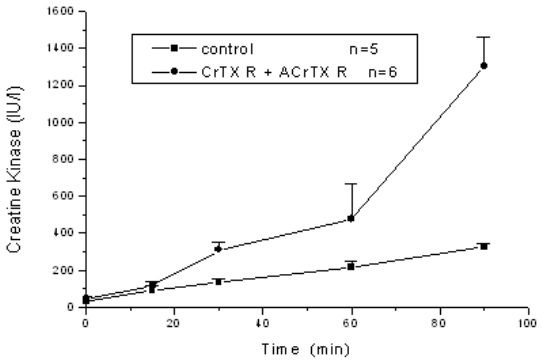
Time (min)

G)


controle
CrTX R + AVTR
Creatino Kinase (UI)
Time (min)

H)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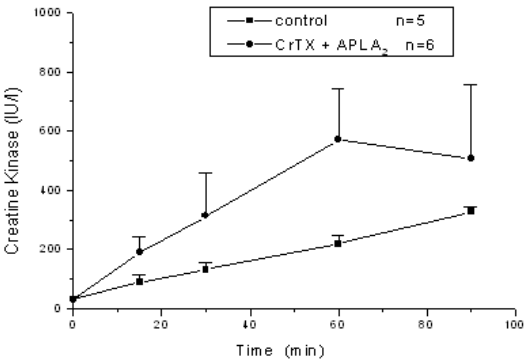
Creatine Kinase (IU/l)
Time (min)

I)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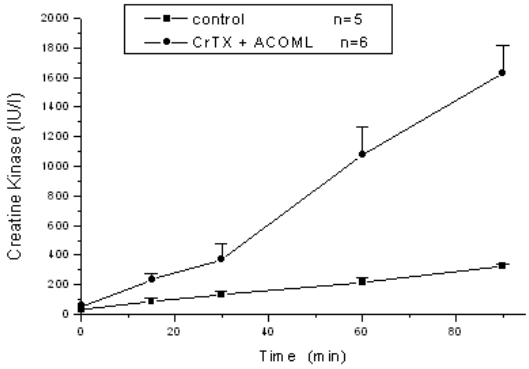
Creatine Kinase (IU/l)
Time (min)

J)


control n=5
CrTX + ACOML n=6
Creatine Kinase (IU/l)
Time (min)

3. Capítulo 1 – conclusões principais:

☛ Os perfis cromatográficos (HPLS SW 300) dos venenos de *C. d. terrificus e C. d. ruruima* revelam a presença de 4 picos sendo eles: convulxina, giroxina, crotoxina e crotamina, enquanto que os dos venenos de *C. d. cascavella e C. d. collilineatus*, apresentam os picos principais com ausência da crotamima, isto é, são crotamina negativos.

☛ Todos os venenos estudados, assim como a fração crotoxina, mostraram-se semelhantes quanto a sua capacidade bloqueadora neuromuscular.

☛ Quanto à neurotoxicidade, o veneno total e a crotoxina foram mais potentes na preparação de ave quando comparado com a preparação de mamífero.

☛ O efeito facilitador neuromuscular induzido pela CrTM dos venenos *C. d. terrificus* e *C. d. ruruima* foi semelhante.

☛ Na associação dos venenos CrTX+CrTM em preparações de aves, o efeito facilitador neuromuscular induzido por estas toxinas foi abolido.

☛ Os resultados aqui apresentados confirmam aqueles descritos na literatura que afirmam que as CrTX estudadas são iminentemente pré sinaptica (VITAL BRASIL, 1971)

☛ Os experimentos realizados com CrTM sozinha e associada mostram que o efeito facilitador e o bloqueador induzido pelas combinações reproduzem os resultados obtidos com o veneno total, isto é, facilitação seguida de bloqueio total, sugerindo que tais efeitos são devido à presença de crotamina e crotoxina, respectivamente.

Capítulo 2 –

☛ O perfil cromatográfico (HPLC – fase reversa μ- bondapack C-18) da crotoxina revela as isoformas de crotapotina e PLA_2 dos venenos *C.d.terrificus, C.d.cascavella*, *C.d. collilineatus.* Para *C.d.terrificus* obteve-se duas isoformas de crotapotina (F5 e F7) e três de PLA_2 (F15, F16 e F17)e para os venenos *C.d.cascavella, C.d. collilineatus* duas de crotapotina (F3 e F4) e uma de PLA_2 (F6).

☛ Em preparações de mamíferos as crotapotinas e PLA_2 não têm diferenças significativas em relação ao controle. O mesmo efeito observou-se em aves para a crotapotina.

☛ O efeito neurotóxico causado pela PLA_2 em aves demonstra que a crotapotina não é necessária para potencializar o efeito bloqueador da resposta contrátil.

☛ A crotapotina e a PLA_2 recombinadas formam o complexo crotoxina em todas as preparações e venenos das serpentes estudadas, causando bloqueio da resposta contrátil.

Capítulo 3 –

☛ Os antivenenos específicos produzidos em coelhos (anti-veneno, anti-crotoxina e anti-PLA_2), apresentaram excelente qualidade imunogênica evidenciada pelos testes de ELISA, e também foram eficientes em neutralizar o bloqueio neuromuscular induzido pelo VT e CrTX de *C. d. ruruima* e PLA_2 *C. d. collilineatus,* nas preparações de aves.

☛ Ficou evidenciada a cinética de liberação de CK para os antivenenos, o veneno e a crotoxina. O aumento dos níveis de CK em alguns testes foram significativos, provavelmente por influência dos anti-venenos. A grande concentração de proteínas presente na solução, devido ao antivenenos pode explicar este resultado.

☛ Evidenciou-se a capacidade neutralizante antiveneno PLA_2, sobre o bloqueio neuromuscular produzido pelos venenos e crotoxina o que sugere qua a PLA_2 possui papel central no mecanismo de neurotoxicidade bem como o envolvimento da atividade catalítica na ação neurotóxica.

☛ O anti-veneno contra PLA_2 de *C. d. collilineatus,* foi eficiente em neutralizar a atividade neurotóxica do veneno e da crotoxina de *C. d. ruruima,* na mesma proporção que os anti-veneno e anti-crotoxina *C.d.ruruima* anti-venenos crotalico comercial.

4. SUMMARY

This work is divided at three moments, the first one if terrificus deals with a comparative study of the effect of the crude venoms of *Crotalus durissus; C. d. ruruima; C. d. cascavella and C. d. collilineatus,* as well as of the crotoxin and the crotamine on the junction neuromuscular. Venoms *C. d. ruruima* was purified for the first time through HPLC in a column Protein Pack sw 300, where it was gotten the crotoxin and the crotamine (the too much venoms already had been purified and the published data). The neurotoxic study nerve-frênico was carried through in two preparations diaphragm of mouse and to chick biventer cervicis.

The experiments carried through in mammals and birds the waited neurotoxic effect in the was gotten concentrations of 10 μg/ml. The crude venoms and the crotoxin of rastnaks *C. d. terrificus* and *C. d. ruruima* in preparations of mammal had produced increase of the followed amplitude of complete blockade of the contractil reply, differently of the venoms and the crotoxin of rastnakes *C. d. cascavella* and *C. d. collilineatus*, that they had caused only complete blockade to neuromuscular. In preparations of birds the four studied venoms and crotoxin had induced complete blockade, however without causing facilitation.

The crotamine-positive, studied in nerve-frenico diaphragm of mouse of snakes *C. d. terrificus* and *C. d. ruruima*, in the concentration of 10 μg/ml, had caused facilitatori effect returning to the 120 after amplitude have controlled min of incubation. When crotoxin being recombined 10 μg/ml and 5 crotamine μg/ml had the increase of the followed amplitude of complete blockade of the contractil reply. In the preparations assayed in birds, fast followed of blockade presented a facilitatori effect to neuromuscular complete.

At as the moment, isoforms of isolated crotapotin and PLA_2 from the crotoxin of the venoms of the rastnakes of *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus*, had been assayed in mammals and birds in the concentrations of 10 μg/ml. Isolated crotapotin and PLA_2 had not caused significant differences in relation to the control in preparations of mammals. The same not occurring with the PLA_2 tested in birds that blockade caused to neuromuscular, effect this not waited. After the recombination (5 μg/ml crotapotin) + (5 μg/ml PLA_2), the crotoxin complex caused blockade to neuromuscular complete in both preparations.

In the third stage of this work, specific antivenoms against crotoxin the crude venoms and of *C. d. ruruima* had been produced and PLA_2 of the poison of *C. d. collilineatus*. The heading of antibodies and the especific of the produced antivenoms had been evaluated by ELISA.

The neutralization of the neurotoxic activity was evaluated in preparations to chick biventer cervicis. The neutralizing capacity of the antivenoms produced in rabbits was comparable to commercial the anticrotalic serum against the venoms and crotoxin in ratio 1:1. The venoms, the crotoxin and the antivenoms, studied had induced significantly different release of CK of the control.

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