In vitro PHARMACOLOGICAL CHARACTERIZATION OF THE EFFECTS OF *Bothrops neuwiedii* VENOM ON GLIOBLASTOMA CELLS

PUJATTI P. B (1).; SOARES M. A (1).; GEOGHEGAN P (2).; GOUVÊA DOS SANTOS R (1).

(1) Lab. Radiobiologia, Centro de Desenvolvimento da Tecnologia Nuclear/CNEN, BH, MG, Brazil, (2) Servicio Inmunoterapéuticos, Centro Nacional de Control de Calidad de Biológicos ANLIS "Dr. Carlos G.Malbrán", Argentina

Glioblastomas (GBM) are neoplasms that display a high degree of vascularity and invasion. The emergence of an metastatic cell subpopulation is promoted by the development of a pattern of integrin receptors. Disintegrins have been identified in venoms of various snakes species and are powerful inhibitors of cancer cell adhesion to several matrices including basement membrane. Metalloproteases (MMP) are also important components of several snake venoms that are responsible for degrading proteins of extracellular matrix (ECM) and have cytotoxic effect on endothelial cells. Some *Bothrops* MMP, like jararhagin and alternagin-C, have been referred to inhibit melanoma cells growth and metastases induced in experimental mice models. The aim of the present work was to identify and characterize the pharmacological effects of *Bothrops neuwiedii* (BN) venom on cultured murine glioblastomas cells (RT2). RT2 cells were sensitive to the treatment with BN and the effects were dose-dependent. In addition, concentrations of BN higher than 0.75mg/mL induced rounded cell shape and inhibited cell adhesion. However, rounded cells were viable upon reculture and proliferated. In order to shed some ligth on the mechanisms of these effects we evaluated BN proteolytic activity, using gelatin as the substrate. BN catalyzed the hydrolysis of gelatin with optimal pH of 7.2. Take together our results and the role of matrix protein for adhesion and cell survival, the anti-tumoral effects of BN on GBM as well as its effects on cells adherence reported can be ascribed, at least partially, to the involvement of metalloproteases of this venom. Experiments are in development to identify the specific component of the venoms involved in the anti-tumoral activity and further characterize its mechanism of action.

KEY WORDS: *Bothrops* venom, RT2 cell, antitumor

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CORRESPONDENCE TO: RG Santos, Lab de Radiobiologia; CDTN/CNEN; C. P. 941; 30123-970; BH- MG – Brasil, santosr@cdtn.br

BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF AN L-AMINO ACID OXIDASE ISOLATED FROM *Bothrops atrox* SNAKE VENOM

ALVES R. M. (1), ANTONUCCI-PASSOS G. A. (1), MENDONÇA-FRANQUEIRO E. P. (1), CINTRA A. C. O. (1), FRANCO J.J.(1), SAMPAIO S.V. (1).

(1) Faculdade de Ciências Farmacêuticas de Ribeirão Preto, FCFRP, Universidade de São Paulo, USP, Ribeirão Preto-SP, Brazil.

Bothrops atrox is a snake responsible for many cases of ophidic accidents in the Amazonian region (Brazil). It´s venom is a complex misture of proteins, enzymes and peptides. The enzyme L-amino acid oxidase (LAAO) from *Bothrops atrox* (*B. atrox*) snake venom was purified using a combination of molecular exclusion, ion exchange and affinity chromatography steps. LAAO from *B. atrox* is an homodimeric acid glycoprotein (approximate Mr and pI of 67,000 and 4.4 , respectively) and displays high specificity toward hydrofobic amino acids. This protein presented a high sequence homology with other LAAOs from: *Bothrops* spp, *Crotalus* spp, *Calloselasma rhodostoma*, *Agkistrodon* spp, *Trimeresurus stejnegeri*, *Oxyuramus scutellatus* and *Notechis scutellatus*. LAAO from *B. atrox* induces low mouse paw edema when compared wiht crude venom, platelet aggregation with approximately 70% effect of LAAO (10µg/mL) on platelet aggregation but does not show any hemorrhagic activity. This protein displays cytotoxic activity in T cells (JURKAT) with cells death of approximately by 80%, melanoma murine (B16F10) with cells death of approximately 70% and in rat pheochromocytoma cells (PC12) with cells death approximately 70%. These cytotoxic effects occur by apoptotycs mechanisms. LAAO from *B. atrox* does not display cytotoxic activity in mononucleares cells (normal cells). Thus, LAAO from *B. atrox* is a multifunctional protein with promising biotechnological and medical applications.

KEY-WORDS: LAAO, *Bothrops atrox*, anti-tumoral activity.

CORRESPONDENCE TO: RAQUEL DE MELO ALVES, Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP. Departamento de Análises Clínicas, Toxicológicas e Bromatológicas,. Phone: (016) 3602-4287. Fax: (16) 3633-3066. E-mail: rackmelo@fcfrp.usp.br

PURIFICATION AND CHARACTERIZATION OF A NEW ACIDIC PHOSPHOLIPASE A_2 OF *Bothrops pauloensis* SNAKE VENOM

RODRIGUES. R.S. (1), IZIDORO. L.F.M. (1), HAMAGUCHI. A. (1), SELISTRE-DE-ARAÚJO. H.S. (2), HOMSI-BRANDEBURGO. M.I. (1), SOARES. A. M. (3), RODRIGUES. V.M. (1).

(1) Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia; (2) Centro de Ciências Biológicas e da Saúde, Departamento de Ciências Fisiológicas, Universidade Federal de São Carlos, (3) Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto.

Phospholipases A_2 (PLA_2) is a small enzyme that present molecular mass around 15kDa and are found in a variety of biological and cellular fluids as fluid sinovial, macrophages, platelet, pancreatic tissue, among others. The present work describes the purification and chemical, biological and enzymatic characterization of an acidic phospholipase A_2 of *Bothrops pauloensis* snake venom. New fosfolipase A_2 was called BP-PLA_2, this enzyme was purified by ionic exchange followed for hidrophobic chromatography and finishing in reverse-phase column. BP-PLA2 consists of a protein of 15.8kDa and isoelectric point of 4.34. The N-terminal sequences of the enzyme displayed a significant homology with the Asp-49 acid of other snake venoms in first fifteen amino acids. The catalytic activity of the BP-PLA_2 was 315 U/mg, showing a considerable increase of activity PLA_2 and was capable to induce high indirect hemolytic activity in the different intervals of time. Acidic phospholipase A_2 was capable to inhibit platelet aggregation at the presence of collagen, fact not occurred when this enzyme was incubated with ADP, showed a sufficiently sharp edematogenic activity in paw mice. BP-PLA_2 induced miotoxic effect, being the characterization carried through for creatine kinase levels and morphological analyses. These studies had indicated an increase of the creatine kinase levels in 1 hour and the histological analyses had indicated that the PLA_2 induced an intense edema with evident leucocitary infiltration and damage in the muscular cells after 24 hours of injection of the toxin.

KEY WORDS: *Bothrops pauloensis*, snake venom, acidic phospholipase A_2

FINANCIAL SUPPORT: CNPq, CAPES, UFU.

CORRESPONDENCE TO: RENATA SANTOS RODRIGUES, LABORATÓRIO DE TOXINAS ANIMAIS E INIBIDORES. UFU 2E SALA 32. UMUARAMA. 38400-902. (34)3218-2203 R: 22. rsantos.rodrigues@gmail.com

EFFECT OF ANABOLIC STEROID UPON SKELETAL MUSCLE DEGENERATION AND REGENERATION AFTER INJECTION OF *Bothrops jararacussu* VENOM

SOUZA R. W.A.(1), GONÇALVES W.(2), DAL PAI-SILVA M. (2), GALLACCI M. (1).

(1) Department of Pharmacology, Institute of Bioscience, University Estadual Paulista, Botucatu, SP, Brazil. (2) Department of Morphology, Institute of Bioscience, University Estadual Paulista, Botucatu, SP, Brazil.

Myonecrosis and permanent loss of muscle mass is a relevant local toxic effect following envenoming by *Bothrops jararacussu* snake venom. Regeneration of adult skeletal muscle involves the activation, proliferation and differentiation of satellite cells and there are evidences that androgenic anabolic steroids (AAS) increase the proliferation of satellite cell *in vitro*. In this work we evaluated the influence of AAS treatment upon the myonecrosis and the muscle regeneration following envenoming by *Bothrops jararacussu* snake venom. Mice (20-25 g, n = 5) were inoculated with *Bothrops jararacussu* venom (30 µg/50 µl of saline solution) just over the extensor digitorum longus (EDL) muscle of the right posterior limb. During muscle regeneration (21 days), mice received subcutaneous injection of either peanut oil (CON) or nandrolone (NAN 2 and 6 mg/kg) after 12 hour, 7 and 14 days of the venom administration. The histological changes in EDL at 1, 3, 7, 14 and 21 days after the injection of the venom were analyzed by light microscopy. In each group the normal and regenerated muscle fibers were quantified using an image computer program (Qwin, Leica). After 3 and 7 days from the venom injection, several myotubes were identified in the muscle regeneration area and after 21 days EDL muscle showed the majority fibers with normal aspects, round and with central nuclei. EDL muscle that received nandrolone (6 mg/kg) showed a great number of myotubes after 3 and 7 days from the venom injection and normal fibers with peripheral nuclei after 21 days. Nandrolone (2mg/kg) did not improve the muscle regeneration. The results showed that nandrolone (6mg/kg) contributed for the muscle regeneration.

KEY WORDS: *Bothrops jararacussu*; nandrolone, skeletal muscle regeneration.

FINANCIAL SUPPORT: FAPESP, CNPq.

CORRESPONDENCE TO: gallacci@ibb.unesp.br

YOUNG OVINES AS PRODUCERS OF CROTALIC ANTISERUM FROM COBALT-60 IRRADIATED VENOM

FERREIRA JUNIOR R. S. (1, 2), NASCIMENTO N. (3), COUTO R. (4), ALVES J. B. (3), MEIRA D. A. (1), BARRAVIERA B. (1, 2)

(1) Department of Tropical Diseases, Botucatu School of Medicine, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil; (2) Center for the Study of Venoms and Venomous Animals, CEVAP, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil; (3) Radiobiology Supervision - Nuclear Energy Research Institute (IPEN/CNEN/SP), São Paulo, Brazil; (4) Clinical Laboratory of Veterinary, School of Veterinary Medicine and Animal Husbandry, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.

The ELISA technique was used to evaluate and compare young ovine humoral immune response during crotalic antiserum production. Animals were clinically evaluated throughout this process, and the neutralizing capacity of antisera raised against natural (NV) and Cobalt-60 irradiated (IrV) venoms of *Crotalus durissus terrificus* (C.d.t.) was verified by means of in vitro challenges. Three groups of six animals each were used: G1 received NV; G2 was inoculated with IrV; and G3 was used as control. Animals received six immunizations during 84 days at 14-day intervals. ELISA of antibody profile showed significant difference ($p<5\%$) between experimental groups (G1<G2). The neutralizing capacity of antiserum raised against IrV was fivefold higher than that of antiserum raised against NV. Results showed a new possibility of using ovines to produce commercial crotalic antiserum, which may be employed in the treatment of human and animal envenomation. Production cost might be reduced by the subsequent utilization of hyperimmunized ovines as food.

KEY WORDS: *Crotalus durissus terrificus*; hyperimmunization; ovines; crotalic antiserum; irradiation.

FINANCIAL SUPPORT: FUNDUNESP

CORRESPONDENCE TO: R.S. Ferreira Junior. Centro de Estudos de Venenos e Animais Peçonhentos, CEVAP-UNESP. Fazenda Experimental Lageado, Rua José Barbosa de Barros, n 1780. CEP: 18610-307 – Botucatu, São Paulo – Brasil. Fone/Fax: 55-14-38145555. rseabra@cevap.org.br

LABORATORY EVALUATION OF YOUNG OVINES INOCULATED WITH NATURAL OR ^{60}CO-IRRADIATED *Crotalus durissus terrificus* VENOM DURING HYPERIMMUNIZATION PROCESS

FERREIRA JUNIOR R. S. (1, 2), NASCIMENTO N. (3), COUTO R. (4), ALVES J. B. (3), MEIRA D. A. (1), BARRAVIERA B. (1, 2)

(1) Department of Tropical Diseases, Botucatu School of Medicine, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil; (2) Center for the Study of Venoms and Venomous Animals, CEVAP, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil; (3) Radiobiology Supervision - Nuclear Energy Research Institute (IPEN/CNEN/SP), São Paulo, Brazil; (4) Clinical Laboratory of Veterinary, School of Veterinary Medicine and Animal Husbandry, São Paulo State University, UNESP, Botucatu, São Paulo, Brazil.

Laboratory profile of young ovines was studied in order to evaluate and compare their production of antiserum from natural and Cobalt-60 irradiated *Crotalus durissus terrificus* venoms. The parameters analyzed included complete blood count, and urea, creatinine, aspartate aminotransferase, total proteins, albumin and globulin dosages. During the experimental period, animals were fortnightly weighed. According to clinical and weight evaluation, ovines in post-weaning phase showed no changes in their physiological profiles but had excellent weight gain. The parameters analyzed were not statistically different ($p<5\%$) among the groups tested. Results bring a new possibility of utilizing ovines in the commercial production of anticrotalic serum, which may be used to treat human and animal envenomation. Its production cost may be reduced by subsequent use of hyperimmunized ovines as food.

KEY WORDS: *Crotalus durissus terrificus*, hyperimmunization, ovines, anticrotalic serum, irradiation.

FINANCIAL SUPPORT: FUNDUNESP

CORRESPONDENCE TO: RUI SEABRA FERREIRA JÚNIOR, Centro de Estudos de Venenos e Animais Peçonhentos – CEVAP/UNESP, Distrito de Rubião Júnior, s/n, 18618-000, Botucatu, SP, Brasil. Email: rseabra@cevap.org.br

IDENTIFICATION OF PROTEINS FROM *Bothrops jararaca* VENOM INTERACTING WITH HUMAN PROTHROMBIN AND THROMBIN USING PROTEOMIC APPROACH

BEGHINI D.G., RAMOS-GUIMARÃES P., OLIVEIRA-CARVALHO A.L., DUTRA, D.L.S., GUIMARÃES-GOMES V., ZINGALI, RB.

Rede Proteômica do Rio de Janeiro; Instituto de Bioquímica Médica, CCS, UFRJ

The identification of proteins that interact with hemostatic system molecules is a strategy of great importance for the discovery of new compounds with potential therapeutic use. The aim of this work is to identify proteins from *Bothrops jararaca* venom interacting with purified prothrombin and thrombin, from human plasma, through a proteomic approach. Prothrombin and thrombin, purified of the human plasma were incorporate into a 5 ml CNBr - Sepharose 4 B column and *B. jararaca* venom (20 mg and 100 mg) was applied respectively in the affinity columns. The fractions retained in these columns were eluted, dialyzed, concentrated and applied (250 µg) into two-dimensional (2D) gel (pH 3-10) for isoelectrofoccusing and 12,5 % for SDS-PAGE stained with Colloidal Coomassie. Approximately 15-30 spots were detected in each gel, excised and hydrolyzed with trypsin and then analyzed by MS and MS-MS experiments, with MALDI-TOF (VoyagerDE PRO-Applied Biosystems) and MALDI TOF-TOF (ABI 4700 Proteomics Analyser Applied Biosystems). Different classes of proteins were identified such as metalloproteinases (jararagin), serino-proteinase (platelet-aggregating proteinase PA-BJ) and lectins. We also identified in *B. Jararaca* venom a prothrombin activator metalloproteinase with low molecular weight homologue to insularinase from *B. insularis*. The formation of the complex between the Bj galactose C-type lectin and prothrombin was confirmed by native gel electrophoresis and gel filtration chromatography. We concluded that the combination of affinity chromatography with the proteomic technique is efficient in identifying venom proteins that interact with blood components leading to the discovery of new protein x protein interactions. Also these results can guide to a new understanding of the envenomation process.

KEY WORDS: Proteomics, snake venoms; c-type-lectin

FINANCIAL SUPPORT: FAPERJ and CNPq

CORRESPONDENCE TO: RB Zingali, Instituto de Bioquímica Médica, CCS Bl H2 sl 04 Cidade Universitária 21941-590, Rio de Janeiro, Brasil. Phone and Fax: + 55 21 2562 6782 . Email: lzingali@bioqmed.ufrj.br

Bothrops jararaca VENOM PREDICTIVE PROTEOME AND AN OVERVIEW OF C-TYPE LECTIN SUPERFAMILY

GUIMARÃES-RAMOS P.; OLIVEIRA-CARVALHO A.L.; DUTRA D.L.; ZINGALI, R.B.

Rede Proteômica do Rio de Janeiro; Instituto de Bioquímica Médica, CCS, UFRJ

Venoms from *Bothrops* genus snakes are recognized by their action on hemostatic system. Metalloproteases, serineproteases and lectins are some of the molecules responsible for this effect. The aim of this study is to analyze the C-type lectin-like protein family of *Bothrops jararaca* venom, and to contribute understanding their structure-function relationship using proteomics technologies. These proteins from *Bothrops* venoms exist as disulfide-linked $\alpha\beta$ dimers of about 15 kDa. Bothrops jararaca venom was submitted to a two-dimensional (2D) gel (pIs 4-7; MM 12-70 kDa); with or without 100 mM DTT. Treatment with DTT showed a decrease of proteins around 30 kDa and an increase in 15 kDa region. The high molecular weight region had 72 proteins, DTT reduced this number to 34, which shows that they are constituted by disulfide-bonds. After excised these spots were treated with DTT (10mM) and Iodoacetamide (55mM) and then analyzed by MS and MS-MS experiments, with MALDI-TOF (VoyagerDE-PRO Applied Biosystems) and MALDI TOF-TOF (ABI 4700 Proteomics Analyzer Applied Biosystems) respectively. We could identify C-Type Lectin, GPIb α and β chains and other C-Type Lectin-like proteins. These results are in agreement with preliminary imunochemicals assays, when we used serum against bothrojaracin, a protein of the C-type lectin-like family, serum on a Western blotting analysis where a 2D gel prepared in the absence of DTT was transferred to a PVDF membrane, and then at least 10 proteins of 30 kDa region were recognized as being part of that protein family. Our data suggest that it is possible to systematically study multimeric proteins linked by disulfide bond with mass spectrometry technology and 2D gel approach. We will concentrate our efforts in order to identify which subunits are linked by disulfide bonds, and elucidate their isoforms using MS, MS-MS and bioinformatic tools.

KEY WORDS: Proteomics, snake venoms; c-type-lectin

FINANCIAL SUPPORT: FAPERJ and CNPq

CORRESPONDENCE TO:RB Zingali, Instituto de Bioquímica Médica, CCS Bloco H2 sala 04 Cidade Universitária 21941-590, Rio de Janeiro, Brasil. Phone and Fax: + 55 21 2562 6782 . Email: lzingali@bioqmed.ufrj.br

THE INFLUENCE OF A LOW CONCENTRATION OF HEPARIN ON THE MYOTOXICITY INDUCED BY *Bothrops jararacussu* VENOM AND BOTHROPSTOXIN-I

ROSTELATO-FERREIRA S. (1), LEITE G. B. (1), CRUZ-HÖFLING M. A. (2), OSHIMA-FRANCO Y. (1), RODRIGUES-SIMIONI L. (1)

(1) Department of Pharmacology, Faculty of Medical Sciences and (2) Department Histology and Embrylogy, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

Envenomation by snakes of the genus *Bothrops* results in local myonecrosis that is only poorly neutralized by traditional antivenom therapy. This poor neutralization has led to a search for alternative treatments for myonecrosis. The aim of this work was to examine the ability of a low concentration of heparin to attenuate the neurotoxicity and myotoxicity of *Bothrops jararacussu* venom (Bjssu) and its main toxin, bothropstoxin-I (BthTX-I). Neuromuscular activity was assayed in mouse phrenic nerve-diaphragm muscle preparations. Controls preparations were incubated with Tyrode solution, heparin (1 µl/ml) or commercial bothropic antivenom (CBA; 120 µl/ml), whereas treated preparations were incubated with Bjssu or BthTX-I (40 µg/ml). For the neutralization assays, mixtures of Bjssu + heparin or BthTX-I + heparin (40 µg/ml:1 µl/ml), or Bjssu + CBA or BthTX-I + CBA (1:3, w/v) were preincubated for 30 min at 37^{o}C prior to assaying the residual neuromuscular activity. At the end of the experiments, the preparations were fixed in Bouin solution and processed for analysis by light microscopy. Heparin and CBA neutralized the neurotoxicity induced by Bjssu and BthTX-I by 79.6 ± 5.9% (n=6), 78.8 ± 6.8% (n=8), 68.3 ± 6.2% (n=6) and 62.3 ± 6.1% (n=6) for Bjssu + heparin, BthTX-I + heparin, Bjssu + CBA and BthTX-I + CBA, respectively. The corresponding percentages for protection by heparin and CBA against the myotoxicity caused by Bjssu and BthTX-I were 94.4 ± 1.5%, 93.6 ± 2.9%, 97.2 ± 0.6% and 98.5 ± 0.6%, respectively. These results show that a low concentration of heparin was effective in protecting against the neurotoxicity and myotoxicity of Bjssu and BthTX-I.

KEY WORDS: antivenom, *Bothrops jararacussu* venom, bothropstoxin-I, heparin

FINANCIAL SUPPORT: CNPq, FAEPEX/UNICAMP.

CORRESPONDENCE TO: LÉA RODRIGUES-SIMIONI, Department of Pharmacology, UNICAMP, SP, Brazil Phone: + 55 19 3521-9533; Fax: + 55 19 3289-2968; E-mail: simioni@unicamp.br

THE TWITCH POTENTIATION INDUCED BY CROTAMINE HOMOLOGS FROM *Crotalus durissus ruruima* AND C*.d.terrificus* venoms

HERNANDEZ-OLIVEIRA S (1, 3), SUSINE-OLIVEIRA S (1), BORJA-OLIVEIRA C.R (1), LEITE G.B (1) , MARANGONI S (2), HYSLOP S (1), RODRIGUES-SIMIONI L (1).

(1), Department of Pharmacology, Facult of Medical Sciences and (2)Department of Biochemistry, Institute of Biology, State University of Campinas, SP, Brazil; (3) Paulista University – UNIP, Campinas, SP, Brazil.

In this work, we compared the neuromuscular activities of *Crotalus durissus terrificus* (Cdt), *C. d. ruruima* (Cdr), *C. d. cascavella* (Cdc), and *C. d. collilineatus* (Cdcoll) venoms and their respective crotoxin (CrTX) homologs,besides crotamine (CrTM) homologs from Cdt and Cdr (crotamine-positive venoms),in indirectly stimulated mouse phrenic nerve-diaphragm and chick biventer cervicis preparations. CrTX and CrTM were isolated from cdr venom by chromatography on Sephadex G-75 followed by reverse-phase HPLC. All of the venoms and their CrTX homologs (10 mg/ml each) caused complete neuromuscular blockade within 120 min in both preparations. However, only Cdt and Cdr venoms (10 mg/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. CrTM (10 mg/ml) from Cdt and Cdr venoms 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). The facilitation were not significantly different when CrTX was added with CrTM. In mouse and chick preparations, CrTX from the four subspecies caused complete and irreversible neuromuscular blockade within 60-70 min and 15-30 min, respectively. None of the crude venoms or CrTX and CrTM homologs inhibited Ach or KCl induced contractures in chick preparations. Since Cdt and Cdr are the only CrTM-positive venoms within the studied venoms, we suggest that the pronounced and initial facilitatory effect of these venoms is induced by CrTM. In addition, these results show that crotoxin homologs from studied venoms and CrTM homologs from Cdt and Cdr have very similar neuromuscular effects. We conclude that the twitch potentiation followed by neuromuscular blockade caused by the crude venom studied, wasdue the presence of CrTM.

KEY WORDS: HPLC; PLA_2; crotamine

FINANCIAL SUPPORT:CNPq, FAEPEX/UNICAMP

CORRESPONDENCE TO: simioni@unicamp.br

NEUROTOXICITY OF A NEW PHOSPHOLIPASE A_2 FROM *Crotalus durissus ruruima* VENOM

HERNANDEZ-OLIVEIRA S. (1,3), SUSINE-OLIVEIRA S. (1), LEITE G.B. (1), MARANGONI S. (2), HYSLOP S. (1), RODRIGUES-SIMIONI L. (1)

(1) Dep. of Pharmacology, Faculty of Medical Sciences, (2) Dep. of Biochemistry, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil. (3) Paulista University (UNIP), Campinas, SP, Brazil.

Crotoxin, the major toxin in South American rattlesnake (*Crotalus durissus terrificus*) venom, consists of crotapotin, a basic non-enzymatic protein, and a weakly toxic phospholipase A_2 (PLA_2). Crotapotin acts as a chaperon protein that enhances the neurotoxicity of the PLA_2. In this work, we isolated crotoxin from *Crotalus durissus ruruima* venom and studied the neurotoxicity of its subunits. Crotoxin and its subunits were purified by reverse-phase HPLC and yielded two isoforms of crotapotin (CRTp1 and CRTp2) and PLA_2 (PLA_2a and PLA_2b), with molecular masses of 9 kDa and 15 kDa, respectively (by Tricine-SDS-PAGE). The neurotoxicity of these proteins was tested in indirectly stimulated chick biventer cervicis and mouse phrenic nerve-diaphragm preparations mounted in a 5 ml organ bath containing aerated (95% O_2 and 5% CO_2) Krebs or Tyrode solution at 37ºC. When the crotapotin (CRTp2) and PLA_2 isoforms were tested in mouse preparations, only PLA_2b (10 mg/ml, n=4) produced neuromuscular blockade (~30%) after 120 min. In contrast, PLA_2b (10 mg/ml, n=6) was highly neurotoxic in chick preparations, with 50% blockade occurring in 57±3 min (mean+SEM, n=6). The combination of PLA_2b+CRTp2 produced 50% blockade in 79±2 min (n=6) and 19±1 min (n=6) in mouse and chick preparations, respectively. These results show that chick preparations are more sensitive than mouse preparations to crotoxin from *C. d. ruruima* venom, and that, in the absence of crotapotin, PLA_2 causes potent neuromuscular blockade in chick, but not in mouse, preparations.

KEY WORDS: *Crotalus durissus ruruima*, crotoxin, neurotransmission, neuromuscular blockade, phospholipase A_2, South American rattlesnake

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CORRESPONDENCE TO: LÉA RODRIGUES-SIMIONI, Department of Pharmacology, Faculty of Medical Sciences, State Unversity of Campinas (UNICAMP), PO Box 6111, 13083-970, Campinas, SP, Brazil. Tel. +55-19-3521-9534, Fax: +55-19-3289-2968, E-mail: simioni@unicamp.br

GEOGRAPHICAL DISTRIBUTION OF CROTAMINE IN RATTLESNAKE *Crotalus durissus collilineatus* IN THE GOIÁS STATE

AY MORE S., MACEDO J. K. A., PAULA L. L., TAVARES T. O., GUIMARÃES T. V. C., FREITAS R. F., PERINI E. A., BARBOSA C. R., MAGALHÃES M. R.

Centro de Estudos e Pesquisa Biológicas / Universidade Católica de Goiás

Crotamine is a basic myotoxin that when injected in mice cause contractions or paralysis the hind limbs. This miotoxin may be present or not within a same species/subspecies of rattlesnake and it permits the mapping of populations of a certain region. The venom of rattlesnake (*Crotalus durissus collilineatus*) from 42 municipalities in the state Goiás was analyzed through SDS-PAGE procedure to assess their distribution of this species throughout the state. Of the 220 samples collected only 34 were positive for crotamine, 20 of which were females,13 males. Since the number of samples per municipality is small, we were not able to infer a pattern of geographical or sexual distribution, as well as the distribution of the populations per sex. However, up to now crotamine appears to be present in the south-central and southwestern part of the state of Goiás and there does not appear to be any sexual characteristic in the expression of the myotoxin.

KEY WORDS: *Crotalus*, venom, myotoxin, crotamine

CORRESPONDENCE TO: Marta Regina Magalhães, Centro de Estudos e Pesquisas Biológicas, Avenida Universitária 1069, Setor Universitário, CP 86, CEP 74605-010, Goiânia, Goiás, Brasil. reginamaga@gmail.com; aymoresa@gmail.com

EFFECT OF BJUSSUMP-II, A NON-HEMORRHAGIC METALLOPROTEASE FROM *Bothrops jararacussu* SNAKE VENOM, ON PLATELET AGGREGATION AND PRO-CLOTTING ACTIVITY

MARCUSSI S. (1, 2), OLIVEIRA C. Z. (2), SANT'ANA C. D. (2), MENALDO D. L.(2), MAZZI, M. V. (2), FULY, A. L. (3), GIGLIO J. R. (1), SOARES A. M. (2).

(1) Depto Bioquímica e Imunologia, FMRP-USP, Ribeirão Preto-SP and (2) Depto. Análises Clínicas, Toxicológicas e Bromatológicas-FCFRP-USP, Ribeirão Preto-SP, (3) Depto. Biologia Celular e Molecular – Universidade Federal Fluminense, UFF - Niterói, Rio de Janeiro-RJ, Brasil. E-mail: smarcussi@rbi.fmrp.usp.br.

The *Bothrops* envenoming is characterized for inducing intense proteolytic activity, clotting of blood plasma and stimulation of histamine and bradykinin release, resulting in severe local effects such as hemorrhage, edema and necrosis, possibly with direct or indirect participation of metalloproteases. This work objectified the evaluation of the effect of BjussuMP-II, a metalloprotease isolated from *B. jararacussu* snake venom, on platelets, citrated human plasma and different proteic substrates. The BjussuMP-II revealed proteolytic activity on casein, collagen, fibrin, fibrinogen and gelatin, not presenting clotting or anti-clotting effect on citrated plasma, but showing, however, pro-clotting activity. This protease induced 100% of platelet aggregation, losing its activity after addition of PMSF or benzamidine. Thrombolytic agents as the BjussuMP-II can be used in therapies, not only in the control of the fibrinogen synthesis, but also in the dissolution of fibrin clot, being primordial for study and treatment of cardiovascular illnesses. The studies carried on clotting and anti-clotting properties of metalloproteases show the specificity of these enzymes on some blood components, responsible for the haemostatic balance. Currently, the study of the haemostasia using snake venoms has shown that some toxins can also be useful in the diagnosis of haemostatic disturbs.

KEY WORDS: Metalloprotease, snake venom, platelet aggregation, effect on haemostasia.

FINANCIAL SUPPORT: FAPESP, CNPq, CAPES, USP.

CORRESPONDENCE TO: MARCUSSI, SILVANA, FMRP, Departamento de Bioquímica e Imunologia, Ribeirão Preto-SP, Brasil. Fone: + 51 (16) 3602 47 14. Fax: + 51 (16) 3602 47 25. Email: smarcussi@rbi.fmrp.usp.br.

MOLECULAR CHARACTERIZATION OF BJUSSUMP-II, A NEW NON-HEMORRHAGIC METALLOPROTEASE FROM *Bothrops jararacussu* SNAKE VENOM: CDNA CLONING AND SEQUENCE

MARCUSSI S. (1, 2), OLIVEIRA C. Z. (2), SANT'ANA C. D. (2), MENALDO D. L. (2), STÁBELI, R. G. (3), GIGLIO J. R. (1), SOARES A. M. (2).

(1) Depto Bioquímica e Imunologia, FMRP-USP, Ribeirão Preto-SP and (2) Depto. Análises Clínicas, Toxicológicas e Bromatológicas-FCFRP-USP, Ribeirão Preto-SP, (3) IPEPATRO, UNIR, Rondonia-RO, Brasil. E-mail: smarcussi@rbi.fmrp.usp.br.

The group of metalloproteases from snake venoms contains zinc dependent enzymes, responsible for the hemorrhagic effect induced by the majority of the Viperidae snake venoms. This work objectified the cloning and sequencing of cDNA of BjussuMP-II, a non-hemorrhagic metalloprotease isolated from *Bothrops jararacussu* snake venom. This protease showed only one polypeptidic chain with relative molecular weight of 24,000 in the absence and presence of denaturing agents, showing to be monomeric. The cDNA of the protease was obtained from the total RNA extracted from *Bothrops jararacussu* venomous gland, being cloned in *E. coli*, and then extracted and sequenced. The complete sequence of the BjussuMP-II, acquired by molecular biology techniques, disclosed 615 pb that codify for 205 aa. The N-terminal sequence of the first 30 amino acid residues, directly from the purified enzyme, confirmed that this cDNA codifies the same protein. Through the alignment of the BjussuMP-II sequence with sequences of metalloproteases from venoms of different snake species, a great homology could be observed, mainly between metalloproteases of the class P-I. The boarded aspects in this work will be able to bring complementary information on mechanisms of action, relating structure and function, thus resulting in a better understanding of the effects induced by snake venom metalloproteases.

KEY WORDS: Metalloprotease, snake venom, proteic structure.

FINANCIAL SUPPORT: FAPESP, CNPq, CAPES, USP.

CORRESPONDENCE TO: MARCUSSI, SILVANA, Faculdade de Medicina de Ribeirão Preto – FMRP, Departamento de Bioquímica e Imunologia, Ribeirão Preto-SP, Brasil. Fone: + 51 (16) 3602 47 14. Fax: + 51 (16) 3602 47 25. Email: smarcussi@rbi.fmrp.usp.br.

PRELIMINARY DATA ABOUT THE GEOGRAPHIC DISTRIBUTION OF VENOMOUS SNAKES IN THE GOIAS STATE

TAVARES T. O., AY MORÉ S., PAULA L. L., FREITAS R. F., PERINI E. A., MACEDO J. K. A., BARROS J. S., GUIMARÃES T. V. C., PERES M. C. MAGALHÃES M. R.

Centro de Estudos e Pesquisas Biológicas / Universidade Católica de Goiás

The snakes belong to the Squamata order and integrate 2900 species approximately, being that about 321 species occur in Brazil. These, 56 species are venomous. In the Goiás State venomous snakes with medical interest are currently constituted by 7 species, congregated into 3 genus and 2 families. This study verified where the populations of venomous snakes are located in the Goiás States. It was used the data gotten through the raising of NUROG/CEPB/UCG (Núcleo Regional de Ofiologia de Goiânia / Centro de Estudos e Pesquisas Biológicas / Universidade Católica de Goiás) Registers Books during the period of 1990 to 2006. The municipalities were grouped in macroregions (Região Sudeste Goiano, Região Oeste Goiano, Região Metropolitana de Goiânia, Região Sul Goiano, Região Noroeste Goiano, Região Sudoeste Goiano, Região Nordeste Goiano, Região Norte Goiano and Região Entorno do Distrito Federal). According to the analyzed registers the specie *Crotalus durissus collilineatus* (67.7%), represented the biggest amount of specimens, followed by *Bothrops moojeni* (23.3%), *Bothrops neuwiedi* (7.2%), *Bothrops alternatus* (0.4%), *Micrurus lemniscatus* (0.2%) and *Micrurus frontalis* (1.0%). The species *Crotalus durissus collilineatus*, *Bothrops neuwiedi, Bothrops moojeni, Micrurus frontalis* and *Micrurus lemniscatus* are distributed uniformly by all Goiás State and the specie *Bothrops alternatus* is distributed only along the Southwestern region.

KEY WORDS: snake venomous, geographical distribution, macroregions, Goiás.

CORRESPONDENCE TO: Marta Regina Magalhães, Centro de Estudos e Pesquisas Biológicas, Avenida Universitária 1069, Setor Universitário, CP 86, CEP 74605-010, Goiânia, Goiás, Brasil. reginamaga@gmail.com; aymoresa@gmail.com

MOLECULAR MECHANISMS GOVERNING THE ANTI-APOPTOTIC EFFECT ON NEUTROPHILS OF VLO5, A HETERODIMERIC VGD/MLD-DISINTEGRIN

SALDANHA-GAMA R, MORAES JA, MARIANO-OLIVEIRA A, COELHO AL, MARCINKIEWICZ C (1), ZINGALI R(2), BARJA-FIDALGO C

Dept. Pharmacology, UERJ(1), Temple Univ, PA, USA(2) IBqM-UFRJ(3), Rio de Janeiro, Brasil

VLO5, is a VGD/MLD heterodimeric disintegrin isolated from *Vipera lepetina* venom, that was shown to be a selective ligand of a9/a4b1 integrin. VLO5 activate *in vitro* human blood neutrophils, inducing focal adhesion kinase activation, cytoskeleton mobilization and chemotaxis Neutrophils (PMN) are short-lived cells and the correct regulation of their apoptotic programme is vital to ensure the maintenance of PMN numbers in the circulation and the efficient removal of invading pathogens and the rapid resolution of the inflammatory response. Integrins mediate dowstream survival signals in different cells, through the activation of intracellular pathways. Once activated, PI3K and MAPK pathways can interfere with the balance between anti- and pro-apoptotic Bcl-2 family members, Bcl-xL and Bad, modulating mitochondrial integrity and preventing cytochrome c release. In the present study, we evaluated the signaling pathways involved in the pro-survival effects of VLO5 on human blood neutrophils. Our data show that, in vitro, probably through interactions with a9/a4b1 integrins, VLO5 activate integrin signaling pathways, potently reducing PMN spontateous apoptosis. These effects are dependent on PI3K and MAPK pathways activation, since LY294002 (PI3K inhibitor) and PD95059 (MAPK inhibitor) revert VLO5 effects. Moreover we show that VLO5 induce the expression of the anti-apoptotic protein Bcl-xL, degradation of the pro-apoptotic protein Bad and prevented mitochondrial membrane damage, inhibiting cytochome c release. In conclusion, as the mechanistic details of the VLO5 and a9/a4b1 integrins interactions on human PMNs become clearer, it should become possible to develop logical combinations of drugs that optimize (or minimize) the susceptibility of selected target cell populations to apoptosis during therapeutic interventions.

KEY WORDS: Disintegrin, neutrophil, apoptosis, intracellular signaling

FINATIAL SUPPORT: CNPq, FAPERJ, CAPES, SR2-UERJ

CORRESPONDENCE TO: C. Barja-Fidalgo. Dept Farmacologia, UERJ. Av 28 de setembro 87 fds, Rio de janeiro 20551-030, RJ, Brasil

UNDERSTANDING THE *in vitro* EFFECTS OF SNAKE VENOMS USING MANGANESE

BUENO L. G. (1), LEITE G. B. (1), ABREU V.A. (1), CRUZ-HÖFLING M. A. (2), RODRIGUES-SIMIONI L. (1), OSHIMA-FRANCO Y. (1).

(1) Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil, (2) Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil.

In this study, we examined the effects of Mn2+, a neuromuscular blocker with pre- and postsynaptic actions, on the neurotoxicity and myotoxicity induced by *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms in chick biventer cervicis preparations. Pretreating the preparations with Mn2+ (0.66 or 1.6 mM) did not affect the blockade induced by *C. d. terrificus* venom or KCl-induced contractures, but partially reduced ACh-induced contractures. On the other hand, both concentrations of Mn2+ partially prevented the blockade induced by *B. jararacussu* venom (seen after washing the preparations with Krebs solution), whereas only 1.6 mM Mn2+ significantly restored ACh-induced contractures. Pretreatment with Mn2+ (1.6 mM) partially prevented the muscle damage and the release of creatine kinase induced by both venoms. These results show that Mn2+ did not prevent the neurotoxicity of Cdt venom, but partially reduced its myotoxicity, whereas this metal partially attenuated the myotoxicity and neuromuscular blockade caused by *B. jararacussu* venom. The pre- and postsynaptic actions of Mn2+ may be useful for studying snake venoms that show one or both of these activities.

KEY WORDS: contracture, divalent cation; myotoxicity; neuromuscular blockade; neurotoxicity, manganese.

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CORRESPONDENCE TO: YOKO OSHIMA-FRANCO. Faculdade de Ciências Médicas Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Tel. +55 19 3521 9533, fax +55 19 3289 2968, E-mail: yofranco@terra.com.br

EFFECTS OF COMMERCIAL AND SPECIFIC ANTIVENOMS AGAINST *Micrurus altirostris* (URUGUAYAN CORAL SNAKE) VENOM

ABREU V. A. (1), LEITE G. B. (1), BORJA-OLIVEIRA C. R. (1), HYSLOP S. (1), FURTADO M. F. D. (2), RODRIGUES-SIMIONI L. (1).

(1) Departamento de Farmacologia, Faculdade de Ciências Médicas Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil. (2) Laboratório de Herpetologia, Instituto Butantan, São Paulo, SP, Brasil.

Micrurus altirostris (Uruguayan coral snake) venom is highly neurotoxic and irreversibly blocks neuromuscular transmission. However, the lethality of M. altirostris venom is not neutralized by commercial antivenom. In this work, we examined the ability of commercial antivenom and antivenom raised in rabbits to neutralize the lethality and neurotoxicity of *M. altirostris* venom. Neutralization *in vivo* was tested in chicks (LD_{50} 0.042 mg/kg, i.m.) and mice (LD_{50} 0.255 mg/kg, i.p.) injected with venom (5 LD50):antivenom mixtures (n=6 animals/group). The venom:antivenom ratios used were 1:1, 1:2.5, 1:5, 1:10 and 1:20, assuming that 1 ml of commercial antivenom neutralized 1.5 mg of *Micrurus* spp. venom (antivenom package insert). The neutralization of venom neurotoxicity was assessed in chick biventer cervicis and mouse phrenic nerve-diaphragm preparations. *Micrurus altirostris* venom (1 mg/ml) produced total neuromuscular blockade in both nerve-muscle preparations. Commercial antivenom and rabbit antivenom showed low efficacy in neutralizing the neurotoxicity and lethality. Commercial failed to neutralize the neuromuscular blockade when tested in the proportion recommended by the manufacturer; a similar result was obtained with rabbit antivenom. Total neutralization of the neuromuscular blockade and lethality was seen only with a venom:antivenom ratio of 1:20. These findings suggest a need for more specific antivenoms to neutralize the activities of Brazilian coral snake species other than *M. corallinus* and *M. frontalis*.

KEY WORDS: chick biventer cervicis, lethality, mouse phrenic nerve-diaphragm, neuromuscular junction.

FINANCIAL SUPPORT: CNPq

CORRESPONDENCE TO: LÉA RODRIGUES-SIMIONI. Faculdade de Ciências Médicas Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Tel. +55 19 3521 9533, fax +55 19 3289 2968, E-mail: simioni@unicamp.br

EFFECTS OF TWO PHOSPHOLIPASES A_2 (PLA_2S) ISOLATED FROM *Bothrops asper* (BA) SNAKE VENOM ON LEUKOCYTES CYCLOOXYGENASES (COXS)

MOREIRA, V. (1), ZAMUNER, S.R. (1), ZULIANI, J.P. (1), WALLACE J. L. (2), GUTIÉRREZ, J.M. (3), TEIXEIRA, C.F.P. (1)

(1) Laboratory of Pharmacology, Butantan Institute, Brazil; (2) University of Calgary, Canada, (3) Instituto Clodomiro Picado, Costa Rica.

Two myotoxins (MTs) with PLA_2 structure were isolated from Ba snake venom: MT-III, a PLA_2-Asp49 with high enzymatic activity and MT-II, a PLA_2-Lys49, devoid of catalytic activity. However both evoke inflammatory events. In this study we evaluated the effects *in vivo* and *in vitro* of these on the expression and activity of the COX-1 and -2, and the release of prostanoids. Male Swiss mice were injected i.p. with MT-II or III (1 µg/g). At selected time, in peritoneal washes, were determined PGE2 and PGD2 concentrations by EIA. Expression of the COXs was determined by western blot and activity by colorimetric assay in leukocytes. MT-II and MT-III induced expression of COX-2 but not COX-1 in peritoneal leukocytes from 1 up to 12 h. The COX-2 showed activity whereas the constitutive COX-1 activity was not modified, by both PLA_2s. Moreover, these MTs increased the levels of PGE2 (from 30 min 12 h) and PGD2 (15 min up to 3 h) in the peritoneal exudates. *In vitro*, incubation of MT-II or III (6.5 µg/mL) with macrophages resulted in increased release of PGE2 and PGD2 and expression of COX-2 but not of COX-1. These data demonstrate the ability of snake venom PLA_2s to induce the expression of active COX-2 protein. This effect may be at least in part due to a direct action of both MTs on macrophages. Induction of expression of COX-2 may be the major mechanism for production of prostanoids induced by both PLA2s. The catalytic activity of some snake PLA_2s may not be relevant for their stimulating effects on COXs.

KEY WORDS: phospholipase A_2; prostaglandins; cyclooxygenases.

FINANCIAL SUPPORT: FAPESP (02/13458-0), CNPq.

CORRESPONDENCE TO: Vanessa Moreira, Instituto Butantan, Laboratório de Farmacologia, São Paulo, Brasil. CEP: 05503-900. Phone: +55 11 37267222 (r. 2093). Email: vanessam@usp.br

Bothrops jararaca (BJV) AND *Crotalus durissus terrificus* (CDTV) VENOMS ELICIT DISTINCT RESPONSES REGARDING TO PRODUCTION OF PROSTAGLANDINS D_2 AND E_2, AND EXPRESSION OF CYCLOOXYGENASES

MOREIRA V. (1), ZAMUNER, S.R. (1), WALLACE J.L. (2), TEIXEIRA C.F.P. (1)

(1) Laboratory of Pharmacology, Butantan Institute, Brazil; (2) University of Calgary, Canada.

Prostaglandins, synthesized by cycloxygenases, play relevant roles in many pathophysiological processes including inflammation and hyperalgesia. In this study the profiles of PGE_2 and PGD_2 production secondary to injection of BjV, with inflammatory activity or CdtV, with antiinflammatory and antinociceptive properties, into mice were evaluated. In addition, the ability of these venoms to induce expression of cyclooxygenases -1 (COX-1) and -2 (COX-2) was investigated. Male Swiss mice were injected i.p. with CdtV (250 mg/kg) or BjV (25 mg/kg). At selected time, in peritoneal washes, were determined PGE2 and PGD2 concentrations by EIA. Expression of COXs was determined by western blot. Intraperitoneal injection of BjV but not of CdtV induced the release of PGD2 at 30 min and of PGE2 from 3 up to 12 h after injection. Moreover, BjV up-regulated expression of COX-2 but not of the COX-1, suggesting that expressed COX-2 is the critical enzyme for prostaglandins production in the late periods of BjV effect. In contrast, CdtV does not have any effect on expression of both COX-1 and -2 proteins. Differences between BjV and CdtV in the ability to regulate prostaglandins synthesis can account distinct effects with regard to inflammation. Moreover, inhibition of COX-2 by selective drugs may be of value to counteract the severe local inflammation induced by BjV in the victims.

KEY WORDS: snake venom; prostaglandins; cyclooxygenases.

FINANCIAL SUPPORT: FAPESP (02/13458-0), CNPq.

CORRESPONDENCE TO: Vanessa Moreira, Instituto Butantan, Laboratório de Farmacologia, São Paulo, Brasil. CEP: 05503-900. Phone: +55 11 37267222 (r. 2093). Email: vanessam@usp.br

NEUTRALIZATION OF SYSTEMIC AND LOCAL TISSUE ALTERATIONS INDUCED BY VIPERIDAE SNAKE VENOMS BY *Schizolobium parahyba* AQUEOUS EXTRACT

MENDES M. M.(1), VALE L. H. F. (1), LOPES D. S. (1), OLIVEIRA C. F. (1), AMAGUCHI A.(1), HOMSI-BRANDEBURGO M. I. (1), RODRIGUES V. M. (1)

(1) Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia

Medicinal plants are source of many pharmacologically active compounds. *Schizolobium parahyba* is used against ophidism. The aim of this work was to investigate the ability of the aqueous extract of *Schizolobium parahyba* (Sp) to neutralize the local tissue damage and some systemic alterations induced by crude venoms (CV) or isolated toxins (Neuw, BnSP7 and PLA2). For neutralization of some systemic and local tissue alterations two different conditions were tested: (i) Preincubation: venoms or toxins were previously incubated with the EV at ratio (1:50 w/w) for 30 min at 37oC; (ii) Treatment: EV of S.p. inoculation at the same site after 15 min of toxins or venoms injections (1:50 and 1:100 w/w). The hemorrhagic activity induced by Bnp and Bm venoms was totally inhibited by Sp when these samples were preincubated. Sp was able to inhibit considerably the hemorrhage induced by Bnp and Bm venoms, when EV 1:50 and 1:100 (w/w) respectively was inoculated at same site after 15 min of venoms injections. The myotoxicity induced by crude venoms or isolated toxins was inhibited by EV at ratios 1:50 and 1:100 by two different experimental conditions confirmed by the decrease of the level of plasma CK and by histological analysis. The S.p. when preincubated with Bnp at 1:50 (w/w) was able to neutralize significantly the systemic alterations induced by Bnp after 6 h. The consumption of fibrinogen and platelet number decrease were inhibited mainly when the mice were treated by EV at 1:100 (w/w) i.m. 15 min later venom injection. In conclusion, our study with extract of *Schizolobium parahyba* has demonstrated some useful activities that support its traditional use against snakebite.

KEY WORDS: Inhibition, vegetal extract, snake venom

FINANCIAL SUPPORT: UFU and CAPES, FAPEMIG.

CORRESPONDENCE TO: Rodrigues, V. M. Instituto de Genética e Bioquímica - Universidade Federal de Uberlândia- Av. Pará, 1720, CEP38400-920, Umuarama, Uberlândia, MG, Brazil. veridiana@ingeb.ufu.br and mirianmmendes@yahoo.com.br

STUDY OF LOCAL TISSUE DAMAGE INDUCED BY MYOTOXINS ISOLATED FROM *Bothrops neuwiedi pauolensis* SNAKE VENOM (JARARACA PINTADA)

OLIVEIRA C. F.(1), LOPES D. S.(1), MENDES M. M.(1), CLISSA P. B. (2), AMAGUCHI A.(1), HOMSI-BRANDEBURGO M. I. (1), RODRIGUES V. M. A.(1).

(1) Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, Brasil, (2) Laboratório de Imunopatologia, Instituto Butantan, São Paulo, Brasil.

Snakebite envenomation constitutes a relevant public health hazard in Latin América. Most accidents are inflicted by species of the genus *Bothrops*. In addition to systemic alterations, these poisonings are characterized by proeminent local tissue damage due to myonecrosis, hemorrhage and edema. Acute muscle damage induced by these venoms is mainly due to myotoxic phospholipases A_2 (PLA_2). This work reports the local tissue damage induced by BnSP-6 and BnSP-7, myotoxins isolated from *Bothrops neuwiedi pauloensis* snake venom. Edematogenic activity was performed using a low-pressure spring (Caliper). Measures of creatine kinase (CK) levels and morfological analysis of BALBc mice gastrocnemius muscle were performed to value the myotoxicicity induced by these myotoxins. Both of them caused oedema in mice paw and increased the release of CK in blood. These alterations were confirmed by the morfological analysis that showed edema, necrosis and inflammation after the firsts hours and regeneration 72 hours after inoculation. Besides, administration of BnSP-6 and BnSP-7 caused a significant increase of cytokines IL-1b, IL-6, IL-8, in the mice footpad, after 3 hours. Therefore, the understanding of the mechanisms of action and the effects caused by these myotoxins allows future farmacological applications, thus contributing to the study and treatment of many kinds of diseases.

KEYWORDS: *Bothrops neuwiedi pauloensis*, myotoxins, BnSP-6, BnSP-7.

FINANCIAL SUPPORT: CNPq, UFU.

CORRESPONDENCE TO: OLIVEIRA, V. M. Instituto de Genética e Bioquímica - Universidade Federal de Uberlândia- Av. Pará, 1720, CEP38400-920, Umuarama, Uberlândia, MG, Brazil. Phone: (34) 32182203 R:22. Email: carolina_udi@yahoo.com.br and veridiana@ingeb.ufu.br

INVESTIGATION OF THE ACTIVITY OF PROTEASES PURIFIED OF THE VENOM OF THE *Bothrops cotiara* (GOMES, 1913) (SERPENTES: VIPERIDAE)

KIZLTYKA V., BARCHIKI F., PEREIRA E. R., PEREIRA L. F., ELIFIO-ESPOSITO S. L.

Laboratório de Fisiologia Animal, CCBS/PUCPR, Curitiba, PR.

Proteases from the crude venom (VB) of *Bothrops cotiara* were partially purified and investigated for proteolytic, hemorrhagic (*in vivo*) and coagulant *(in vitro*) activities. The proteolytic activity was measured using casein as substrate and zimograma in gelatin containing gel. The purification by Sephadex G-100, followed by anion exchange chromatography (DEAE-Sephadex) resulted in three distinct peaks (I, II, III). Of these it was observed that only Peak I showed caseinolytic activity, while that Peak III showed action over gelatin. For hemorrhagic activity initially the Maximum Hemorrhagic Dose was determined (50 mg/50 ml), while for the tests of coagulante activity initially the Minimum Dose Coagulante was established (12,5 mg/ml) and purified fractions were tested in the same concentrations. Peaks II and III demonstrated hemorrhagic activity with 0,65 areas of 1,2 and cm2, respectively. The assays of coagulant activity showed that only peak II showed pro-coagulante activity, with coagulation time of 171,67 ± 5,92 sec. The results suggest the partial purification of a serinoprotease and a metaloprotease.

KEY WORDS: *Bothrops cotiara*, purification, serinoprotease, metaloprotease.

FINANCIAL SUPPORT:PUCPR, CNPq.

CORRESPONDENCE TO: SELENE L. ELIFIO-ESPOSITO, Laboratório de Fisiologia Animal, Curso de Biologia, CCBS, Pontifícia Universidade Católica do Paraná. CEP 80215-901, Curitiba, PR. Tel. 55-41-32712282. Email: selene.e@pucpr.br

EFFECTS OF *Bothrops marajoensis* VENOM ON THE MOUSE AND CHICK NERVE-MUSCLE PREPARATIONS

CAVALCANTE W. L. G. (1), HERNANDEZ-OLIVEIRA S. (1), LEITE G. B. (1), PONCE-SOTO L. A. (2), MARANGONI S. (2), RODRIGUES-SIMIONI L. (1).

(1) Department of Pharmacology, Faculty of Medical Sciences, University of Campinas, Campinas, SP, Brazil; (2) Department of Biochemistry, Institute of Bioscience, University of Campinas, Campinas, SP, Brazil.

The venoms of some *Bothrops* species produce neuromuscular blockade in avian and mammalian nerve-muscle preparations in vitro. In this study, we investigated the neuromuscular activities (neurotoxicity and myotoxicity) of *Bothrops marajoensis* venom (Bmj) in mouse phrenic nerve-diaphragm muscle (PNDp) and chick biventer cervicis muscle preparations (BCp). The preparations were mounted in a 5 ml organ bath (PNDp - Tyrod solution; BCp - Krebs solution), aerated (95 % O2 and 5 % CO2) at 37 ºC for indirectly stimulation. Myotoxicity was assessed by light microscopic analysis. Data (mean ± S.E.M.; n=4-6) were analyzed by ANOVA ($p<0.05$). The Bmj venom produced a time-dependent blockade in all concentrations. The time (minutes) required to produce 50 % of neuromuscular blockade in PNDp was 112.71±2.95 % (5 µg/ml), 101.50±12.02 % (10 µg/ml) and 42.50±6.32 % (20 µg/ml); in BCp, it was 76.99±9.55 % (1 µg/ml), 31.08±3.14 % (5 µg/ml) and 25.34±3.01 % (20 µg/ml). At venom concentration of 1 µg/ml and 5 µg/ml (BCp), the responses to, exogenously applied, KCl (13.4 mM) and acetylcholine (110 µM and 55 µM), after a complete neuromuscular blockade, were unaffected. However, at 20 µg/ml the venom caused an inhibition of acetylcholine (83.98±2.17 %) and KCl (82.73±4.26 %) responses. Bmj venom (20 mg/ml) induced a light morphological changes corresponding to 26.75± 3.65 % on PNDp muscle fibers, after 120 min incubation. These results indicate that Bmj venom is more potent in BCp than is in PNDp. In low concentration (1 µg/ml, 5 µg/ml), the effects of Bmj venom in BCp are compatible with a pre-synaptic action. Fractionation of the crude venom and the characterization of its neurotoxic components are required to further investigate the mechanism of action of the neuromuscular blockade of Bmj crude venom.

KEY WORDS: *Bothrops marajoensis*; neuromuscular junction; neurotoxic; myotoxic.

FINANCIAL SUPPORT: CNPq.

CORRESPONDENCE TO: simioni@unicamp.br

COMPARISON OF THE NEUROTOXIC EFFECTS OF VENOMS AND CROTOXINS FROM TWO SUBSPECIES OF *Crotalus durissus* SNAKES ON MOUSE NEUROMUSCULAR JUNCTION

CAVALCANTE W. L. G. (1), SANTOS-DIZ-FILHO E. B. (3), CAMPOS T. O. (1), ROMERO-VARGAS F. F. (3), PONCE-SOTO A. L. (3), MARANGONI S. (3), RODRIGUES SIMIONI L. (4), DAL PAI-SILVA M. (2), GALLACCI M. (1).

(1) Department of Pharmacology and (2) Department of Morphology, Institute of Bioscience, University Estadual Paulista, Botucatu, SP, Brazil; (3) Department of Biochemistry, Institute of Bioscience, University of Campinas, Campinas, SP, Brazil; (4) Department of Pharmacology, Faculty of Medical Sciences, University of Campinas, Campinas, SP, Brazil.

The *Crotalus durissus* complex of snakes has a wide distribution in South America. *Crotalus* venoms present a potent neurotoxic activity, which is expressed mainly by crotoxin (CTX), their major toxin. In this study, we compared the neuromuscular activity of venoms from two subspecies of *Crotalus* genus, *C.d. cumanensis* (Cdc) and *C.d. ruruima* (Cdr), on mouse phrenic nerve-diaphragm muscle preparations (PNDp). The preparations were mounted in a 15 ml organ bath with Ringer solution, aerated with carbogen (95% O_2 and 5 % CO_2) at 37 ºC. Paralysing activity was evaluated through the recording of directly and indirectly evoked twitches in PNDp. Data (mean ± S.E.M.; n = 4-6) were analysed by ANOVA ($p<0.05$). Both venoms and their CTX induced an irreversibly and concentration-dependent blockade of indirect twitches. However, only Cdc venom blocked direct contractions. The paralysing activity of Cdc venom was always preceded by a facilitation of both direct and indirect contractions. The time (minutes) required for Cdc venom to produce 50% of blockade (t1/2) of indirect twitches ranged from 87.11±8.8 (1 µg/ml) to 36.47 ± 1.76% (10 µg/ml), and for direct contractions it was 70.5±6.8 (10 µg/ml). For Cdr venom, t1/2 was higher than 90 minutes (5 µg/ml), and it was 53.79 ± 8.58% (10 µg/ml). The data obtained until this moment showed that both venoms presented neurotoxic activities, and these effects could be attributed to their respective CTXs. The present results also indicate that the venom of Cdc is more potent than that of Cdr snake.

KEY WORDS: Crotalidae venom; neuromuscular junction.

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CORRESPONDENCE TO: gallacci@ibb.unesp.br

CROTOXIN INHIBITS NEUROPATHIC PAIN AND THE DEVELOPMENT OF NEUROMAS

CURY Y.(1), NOGUEIRA-NETO F.(1,2), AMORIM R.L.(2), BRIGATTE P. (1), PICOLO G. (1), FERREIRA JR. W.A. (1), NICOLETTI J.L.M.(2)

1-Laboratory of Pathophysiology, Butantan Institute, 2-Faculty of Veterinary Medicine and Zootechny, UNESP, Sao Paulo, Brazil

Crotalus durissus terrificus snake venom (CdtV) induces analgesia mediated by opioid receptors and the factor responsible for this effect, namely crotalphine, was recently isolated and characterized. However, recent data have indicated that crotoxin (CTX), the main neurotoxic component of CdtV, exerts antinociceptive effect in experimental models of acute and cancer pain. The aim of the present study is to further characterize the analgesic action of CTX, evaluating the effect of the toxin on neuropathic pain and determining the mechanisms involved in this effect. For induction of neuropathic pain, the sciatic nerve of rats was exposed and 0.5 cm of the nerve was removed. Pain-related behavior and development of neuromas were analyzed over a 64-day period after surgery. The rat paw pressure test was used for hyperalgesia evaluation. The presence of neuromas was determined by histological analysis of the nerves. Hyperalgesia was detected 2 h after surgery and persisted for 64 days. Few animals developed neuromas until day 7, however 80 % of the rats presented neuromas on day 64. Neuromas often occur at the proximal stump of the transected nerve. CTX (0.01 mM) applied to the proximal and distal nerve stumps, immediately after nerve transection, blocked hyperalgesia. The analgesic effect was observed 2 h after CTX treatment and persisted for 64 days. CTX-induced analgesia was blocked by i.p. administration of atropine (10 mg/kg), or significantly inhibited by i.p. injection of yohimbine (2 mg/kg) and methysergide (5 mg/kg). Atenolol (1 mg/kg) and naloxone (1mg/kg) did not interfere with this analgesic activity. Histological analysis showed that CTX delays and/or significantly inhibits the development of neuromas. The results indicate that CTX induces a long-lasting analgesic effect on neuropathic pain and inhibits the development of the neuropathy. The analgesic effect is mediated by muscarinic receptors, 5HT-receptors and a-adrenoceptors.

KEY WORDS: Crotoxin, analgesia, neuroma, neuropathic pain, muscarinic receptors, *Crotalus durissus terrificus* venom

CORRESPONDENCE: yarac@attglobal.net

EVALUATION OF THE DEVELOPMENT OF DELAYED HYPERALGESIA BY CROTALPHINE, AN ANALGESIC OPIOID OBTAINED FROM *C. d. terrificus* VENOM (CdtV)

PEREIRA L. (1), PICOLO G. (1), BRIGATTE P. (1), CURY Y.(1), Konno K. (2)

(1) Lab. of Pathophysiology, Butantan Institute, (2) Center for Applied Toxinology

Crotalphine (CRP), a peptide obtained from CdtV, induces potent and long-lasting analgesia mediated by opioid receptors. A potential problem with opioid treatment is the paradoxical development of delayed hyperalgesia. The aim of this study is to investigate the development of hyperalgesia after treatment with CRP. Pain threshold was assessed by the rat paw pressure test. Prostaglandin E2 (PGE2, 100 ng/paw) was injected to induce pain sensitization. Rats were treated with CRP (p.o., 20 ng/kg; 10 µg/kg.) or morphine (M, s.c.,1 µg/kg, 1 or 5mg/kg). M (6, 12, 24 µg/paw) was also directly injected in the paw. Naloxone (N, 1 mg/kg), an opioid receptor antagonist, DPDPE (250, 500, 1500 µg/kg), DAMGO (150, 300, 500 µg/kg) and U-50488 (4,5, 9. 18 µmol/kg), δ, µ and k selective opioid receptor agonist, respectively were s.c. administered to evaluate the contribution of opioid receptors to both the analgesic and hyperalgesic effects. Analgesia was observed 3 h after CRP (20 ng and 10 mg/kg) or 1 h after M administration. The analgesic effect of CRP was detected up to 120 h after treatment, when evaluated in the model of PGE2-induced sensitization. Hyperalgesia was detected 96-120 h after M (5 mg/kg) treatment. M, administered in the paw, induced local analgesia, but did not cause delayed hyperalgesia, suggesting that this phenomenon is mediated by central mechanisms. N blocked both the analgesic and hyperalgesic effects of morphine, suggesting the involvement of opioid receptors in both phenomena. Only DAMGO induced analgesia and delayed hyperalgesia (96-144 h), indicating that delayed hyperalgesia is due to activation of m-opioid receptors. These data suggest that CRP, despite presenting opioid activity, does not induce delayed hyperalgesia, being an important candidate for a new opioid drug that fits into the pharmacotherapy of pain.

FINANCIAL SUPPORT: CNPq; CAT/CEPID/FAPESP; COINFAR

KEY WORDS: Crotalphine, *C. d. terrificus* venom, analgesia, hyperalgesia, opioid

CORRESPONDENCE: YARA CURY, Phone: +55 11 37267222. E.mail: yarac@attglobal.net

EFFECT OF CROTOXIN (CTX) ON CIRCULATING LYMPHOCYTES AND LYMPHOID ORGANS

ZAMBELLI V. O. (1), SAMPAIO S. C. (1), BRITTO L. R. G. (2), SPADACCI-MORENA D.D (1), CURY Y. (1)

(1) Laboratório de Fisiopatologia - Instituto Butantan , (2) Departamento de Fisiologia e Biofísica- ICB, USP.

CTX, the main neurotoxin of *C. d. terrificus* venom and its subunit phosfolipase A_2 (PLA_2) inhibit macrophage activity. Lipoxygenase (LO)-derived lipid mediators mediate this effect. Our previous work showed that these toxins promote leukocyte endothelium adhesion and reduce the number of lymphocytes in blood and lymph, but the mechanisms involved in these effects are not known. This study aimed to investigate the effect of CTX and PLA_2 on lymphoid tissue and lymphocyte proliferation, as well as to investigate the involvement of adhesion molecules and LO-derived lipid mediators on the effect of CTX or PLA_2 on circulating lymphocytes. The lymphoid organs of Wistar rats were removed 2h after s.c. injection of CTX (18µg/rat) or PLA_2 (10.4µg/rat). Paraffin-embedded tissue sections were stained with HE for histopathological analysis. The expression of T and B lymphocyte was determined by immunohistochemistry. CTX or PLA_2 reduced (42% and 51%, respectively) the number of circulating lymphocytes 2 h after treatments. Fucoidin and zileuton blocked this inhibitory effect. The toxins promoted, in mesenteric lymph node, both follicular hyperplasia and cells death. Hyperplasia of spleen white pulp was also detected. Toxins treatment increase T and B lymphocyte immunostain and decrease lymphocyte proliferation. These toxins also increase the number of lymphocytes migrating through high endothelial venules (HEVs). These data indicate that CTX and PLA_2 decrease the number of circulating lymphocytes, increase the lymphocyte traffic on HEVs and increase the number of lymphocytes in lymphatic tissue. Despite the increase in the number of lymphocytes in lymphoid tissues, there is an inhibition of lymphocyte function. L- selectin and LO-derived lipid mediators mediate the decrease in the number of circulating lymphocytes.

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KEY WORDS: Crotoxin, lymphocyte, lymphoid organs, snake venom, lipidic mediator, adhesion molecule

CORRESPONDENCE: YARA CURY, Phone: +55 11 37267222. Fax: +55 11 37261505. Email: yarac@attglobal.net