

## $\omega$ -conotoxin MVIIA intralesional injection in spinal cord injury in rats

### Aplicação intralesional da $\omega$ -conotoxina MVIIA no trauma medular em ratos

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#### ABSTRACT

This study aimed to investigate the neuroprotective effect of  $\omega$ -conotoxin MVIIA (MVIIA) intralesional application in rats submitted to spinal cord injury. Male Wistar rats, weighing  $300g \pm 23.4$ , were distributed in five groups: negative control (SHAM), placebo (PLA),  $5\mu M$  MVIIA,  $10\mu M$  MVIIA and  $20\mu M$  MVIIA. After laminectomy of the 12<sup>th</sup> thoracic vertebra (SHAM), the PLA,  $5\mu M$  MVIIA,  $10\mu M$  MVIIA and  $20\mu M$  MVIIA groups were subjected to acute compressive spinal cord trauma for five minutes, and then five minutes later, the animals received specific treatment in a standard total volume of  $2\mu L$ , by intralesional route, using sterile PBS as placebo. Locomotor activity was assayed using Basso Beattie Bresnahan (BBB) scale to show the patterning of SCI. With 48 hours of injury, the animals were euthanized, the liquor sample was collected in atlantooccipital space, and also the spinal segment, including the epicenter and caudal region to injury. Assays were performed for mitochondrial viability, serum glutamate, production of reactive oxygen species (ROS) and lipid peroxidation (LP) were performed. The study design was randomized and the data submitted to ANOVA and comparison of means by SNK test, and data from BBB scale were evaluated using Kruskal-Wallis test ( $P < 0.05$ ). There was no significant difference between groups in BBB scores. The MVIIA did not promote decrease in the levels of glutamate, ROS, LP, and did not preserve the mitochondria in the intralesional application five minutes after spinal cord injury in rats.

**Key words:** MVIIA, cone snail, cell viability, glutamate, reactive oxygen species, lipid peroxidation.

#### RESUMO

Objetivou-se investigar o efeito neuroprotetor da aplicação intralesional da MVIIA em ratos submetidos ao trauma medular. Foram utilizados ratos Wistar, machos, com

peso entre  $300g \pm 23.4$ , distribuídos em cinco grupos: controle negativo (SHAM), placebo (PLA),  $5\mu M$  MVIIA,  $10\mu M$  MVIIA e  $20\mu M$  MVIIA. Após a laminectomia da vértebra torácica 12 (SHAM), os grupos PLA,  $5\mu M$  MVIIA,  $10\mu M$  MVIIA e  $20\mu M$  MVIIA foram submetidos ao trauma medular agudo compressivo por cinco minutos e, cinco minutos após o trauma, receberam o tratamento específico em volume total padrão de  $2\mu L$ , pela via intralesional, sendo utilizado como placebo o PBS estéril. A atividade locomotora foi avaliada pela escala proposta por Basso Beattie Bresnahan (BBB), com intuito de mostrar a padronização do trauma medular. Com 48 horas do trauma, os animais foram submetidos à eutanásia, coletou-se amostra do líquor no espaço atlantooccipital e um segmento medular, incluindo o epicentro e região caudal à lesão. Foram realizados ensaios de viabilidade mitocondrial, dosagem de glutamato, produção de espécies reativas de oxigênio (ERO) e peroxidação lipídica (PL). O delineamento do estudo foi inteiramente casualizado e os dados submetidos ao ANOVA, com comparação de médias pelo teste de SNK e os dados do teste BBB foram comparados utilizando o teste Kruskal-Wallis ( $P < 0.05$ ). Em relação aos escores do BBB, não houve diferença entre os grupos. A MVIIA não promoveu a diminuição dos níveis do glutamato, ERO, PL e não preservou a mitocôndria na aplicação intralesional, cinco minutos após o trauma medular em ratos.

**Palavras-chave:** MVIIA, caramujo marinho, viabilidade celular, glutamato, espécies reativas de oxigênio, peroxidação lipídica.

#### INTRODUCTION

Among the many events that contribute to secondary neuronal death following spinal cord

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injury (SCI), contrasts excitotoxicity mediated by glutamate, excessive accumulation of intracellular calcium with consequent mitochondrial dysfunction and production of reactive oxygen species (ROS). ROS, in turn, react with cellular components, including the lipid plasma membrane, resulting in lipid peroxidation (LP) that leads to membrane rupture and neuronal death (DASURI et al., 2013). Success of SCI treatment depends on how efficiently the secondary injury mechanisms can be altered. So, in an attempt to stop or reduce these injuries, neuroprotective pharmacological strategies have been developed, however they did not show adequate efficiency.

Spinal cord has several voltage-dependent calcium channels (VDCC), with the N and P/Q type involved in the spinal trauma mechanism, and the N type expressed in high concentrations in the dorsal laminae gray matter (GOHIL et al., 1994). Thus, calcium channels blockers have been shown great potential in reducing brain and spinal cord injuries by preventing the intense ion influx and thus the lesion progression. Toxins from cone snails such as  $\omega$ -conotoxin MVIIA or Ziconotide, initially purified as SNX-111, is a potent reversible blocker of N type calcium channels (OLIVERA et al., 1985), capable to inhibit, in synaptosomes, neurotransmitters release such as norepinephrine (BOWERSOX et al., 1995) and glutamate (GONÇAVES et al., 2011), essential in the development of secondary spinal injury. This drug was approved by the Food and Drug Administration in the United States, for analgesic therapeutic use to treat severe chronic and neuropathic cancer pain (SOUZA et al., 2008; BINGHAM et al., 2010).

The MVIIA application has shown significant improvement in traumatic brain injury in rats (VERWEIJ et al., 2000), cerebral ischemia models (VALENTINO et al., 1993; BOWERSOX et al., 1997) and spinal cord ischemia in rats (BURNS et al., 1999). There are no reports in the literature on research in spinal cord injury and MVIIA. Thus, the aim of this study was to evaluate the neuroprotective effects of MVIIA intralésional injection in different doses following SCI.

## MATERIAL AND METHODS

Twenty-five adult, male Wistar rats, weighting  $300\text{g}\pm 23.4$ , were randomly distributed into five groups. Rats were housed in a controlled environment and provided with commercial rodent food and water *ad libitum*. This study was carried out in strict accordance with the recommendations in the

Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

Animals were premedicated with morphine sulfate ( $2.5\text{mg kg}^{-1}$ , subcutaneously), and anesthesia was induced and maintained with isoflurane in a non-rebreathing circuit, through a facemask. The animals were positioned in a stereotactic apparatus, received prophylactic antibiotic with cephalotin ( $60\text{mg kg}^{-1}$ , subcutaneously) and then, prepared for aseptic surgery. An incision was made in the dorsal midline skin and subcutaneous tissue extending from T8 to L1, and the muscle and tissue overlying the spinal column was blunt dissected away revealing the laminae. Using the spiny process of T13 as a landmark, laminectomy of T12 was performed with a pneumatic drill and the lamina was carefully removed to expose the spinal cord. Extradural compression of the spinal cord at the vertebral level of T12 was achieved as previously described (TORRES et al., 2010; OLIVEIRA et al., 2014) for five minutes, using a weight of  $40.5\text{g cm}^{-1}$ . Five minutes later, an intralésional injection was performed according to the experimental group. The incision was closed in two layers. The animals received saline solution ( $15\text{ml kg}^{-1}$ , subcutaneously) and were allowed to recover from anesthesia in a warmed ( $37^\circ\text{C}$ ) box with oxygen therapy.

Post-operative care procedures involved morphine sulfate ( $2.5\text{mg kg}^{-1}$ , orally, every 4 hours) at the surgery day and tramadol chloride ( $5\text{mg kg}^{-1}$ , subcutaneously, every 8 hours) for two days, and cephalexin ( $60\text{mg kg}^{-1}$ , orally, twice daily) and manual expression of the bladder (three times a day) until euthanasia.

The animals were randomly distributed into five groups, with five rats each, according to the treatment protocol: sham-operated (SHAM), placebo (PLA - sterile PBS),  $5\mu\text{M}$  MVIIA,  $10\mu\text{M}$  MVIIA e  $20\mu\text{M}$  MVIIA. MVIIA was prepared in sterile PBS diluting respectively 10, 20 and  $40\text{pmol}$  in a PBS volume of  $2\mu\text{L}$  to reach these final concentrations. In SHAM, the procedure performed was laminectomy. The other groups, five minutes after the trauma, received the specific treatment at the lesion epicenter (intralésional route). This treatment was delivered using  $10\mu\text{L}$  Hamilton syringe attached to a polyethylene tube (PE10) and 30G needle at the rate of  $0.4\mu\text{L min}^{-1}$ .

Side effects evaluation started one day before the surgical procedure for animals conditioning, in the first five hours after application of the toxin and daily until the day of euthanasia. Rats were placed in the open field and evaluated for

15 minutes to the presence of generalized and tail tremor, ranking as absent, mild, moderate and severe.

Beginning three days prior to SCI, animals were allowed to adapt to the open field arena for behavioral testing. Rats were exposed to the open field prior to the surgery and after trauma. During this evaluation, the animals were filmed for four minutes and the videos were later analyzed by two observers who were blinded to animal groups, assigning a score that ranged from zero to 21, using the Basso Beattie Bresnahan (BBB) locomotor rating scale (BASSO et al., 1996) showing the pattern of SCI.

On 48 hours after surgery, the rats were premedicated with xylazine (8mg kg<sup>-1</sup>, intraperitoneally) and deeply anesthetized with an overdose of sodium thiopental (100mg kg<sup>-1</sup>, intraperitoneally). Next, the liquor sample (50-80μL) was collected in atlantooccipital space. Glutamate measurement in liquor was performed enzymatically after the increase in the fluorescence due to the production of NADPH<sup>+</sup> in the presence of glutamate dehydrogenase and NADP<sup>+</sup>. For the assay, was added 1.8ml incubation solution KRH (Krebs Ringer HEPES) without calcium (124mm NaCl, 4mM KCl, 1.2mm MgSO<sub>4</sub>, 10mM HEPES), 5μL of NADP (1mM), 22μL of glutamate dehydrogenase, 5μL of sample and 5μL of standard glutamate. The excitation wavelength was set at 360nm and the emission wavelength to 450nm using a spectrofluorimeter (Shimadzu RF-5301PC, Japan). Glutamate level was calculated as pmol of glutamate per milligram of protein.

Mitochondrial viability was determined by converting 2,3,5-Triphenyltetrazolium (TTC) to the insoluble formazan. The reduction of this compound is dependent on mitochondrial respiratory activity. Therefore, this conversion can be proportional to the number of viable cells. After euthanasia, spinal cord fragment collected from lesion epicenter was subjected to 400mM with the aid of a tissue cutter and after processing in the perfusion chamber, the slices were incubated at 37°C in a solution of 2% TTC for 90 minutes. After this period, the TTC was removed and the tissue washed with 0.9% saline solution and then added 1.5mL of a solution containing dimethylsulfoxide (DMSO) and ethanol in a 1:1 ratio and incubated for 48 hours, at room temperature and protected from light, to solubilize the formazan. The reading was held in a spectrophotometer at 485nm and the results normalized by tissue weight.

Spinal cord fragment was collected from lesion epicenter and dichlorofluoresceindiacetate (DCF-DA) levels were determined as an indicator of the peroxide production of cell components. Fragment

was immediately cooled to -20°C in TRIS-HCl buffer solution (10mM, pH7.4). After homogenization, it was subjected to centrifugation (10000rpm, 5°C, 10min) and an aliquot of 20μL of this supernatant was added to 80μL DCF-DA 125μM. The plate was incubated in triplicate in the dark for 1h at 37°C until the measurement of the fluorescence (488/525nm) in Victor X4 apparatus (Perkin-Elmer). Results were normalized by protein content.

Lipid peroxidation was determined by quantifying the content of malondialdehyde (MDA) present in the homogenate supernatant, also in caudal segment, through the colorimetric reaction with thiobarbituric acid (TBA) at high temperatures. After storage at -20°C, the sample was homogenized in sonicator (Branson Sonifier Model 250) (3 cycles of 4s each) and the homogenate centrifuged at 2500rpm, 4°C for 10min. Pipetted initially 134μL of 0.8% TBA, 134μL of acetic acid buffer (125ml H<sub>2</sub>O; 18,84ml of glacial acetic acid; 5,63ml 12N HCl PA; pH3,4) and 54μL of H<sub>2</sub>O. Upon incubation, 54μL of sample and 54μL of 8.1% SDS (sodium dodecyl sulphate) was added, leaving for 90 minutes at 95°C. The MDA content was measured at a wavelength of 532nm and the results normalized by protein content.

The determination of protein was done by the colorimetric method described by BRADFORD (1976). A volume of 2μL of supernatant, obtained suspending the pellet from caudal segment of the spinal cord, was used for quantification. Samples in triplicate were placed in plates by adding 500μL of 0.15M of NaCl and 500μL of Bradford reagent in each sample. The mixture was incubated for 5 minutes, stirring, and then proceeded to read the absorbance in a spectrophotometer at a wavelength of 595nm. The protein concentration was calculated using a standard curve dilution of 1mg BSA ml<sup>-1</sup> with 1, 3, 5, 7 and 10mg.

All data collected were analyzed using Prism 5 for Windows (GraphPad Software, La Jolla, CA, USA). Data from BBB locomotor rating scale were evaluated using Kruskal-Wallis test. Other data were submitted to analyses of variance, and means were compared using Student-Newman-Keuls test. For all analyses, P value <0.05 was considered statistically significant.

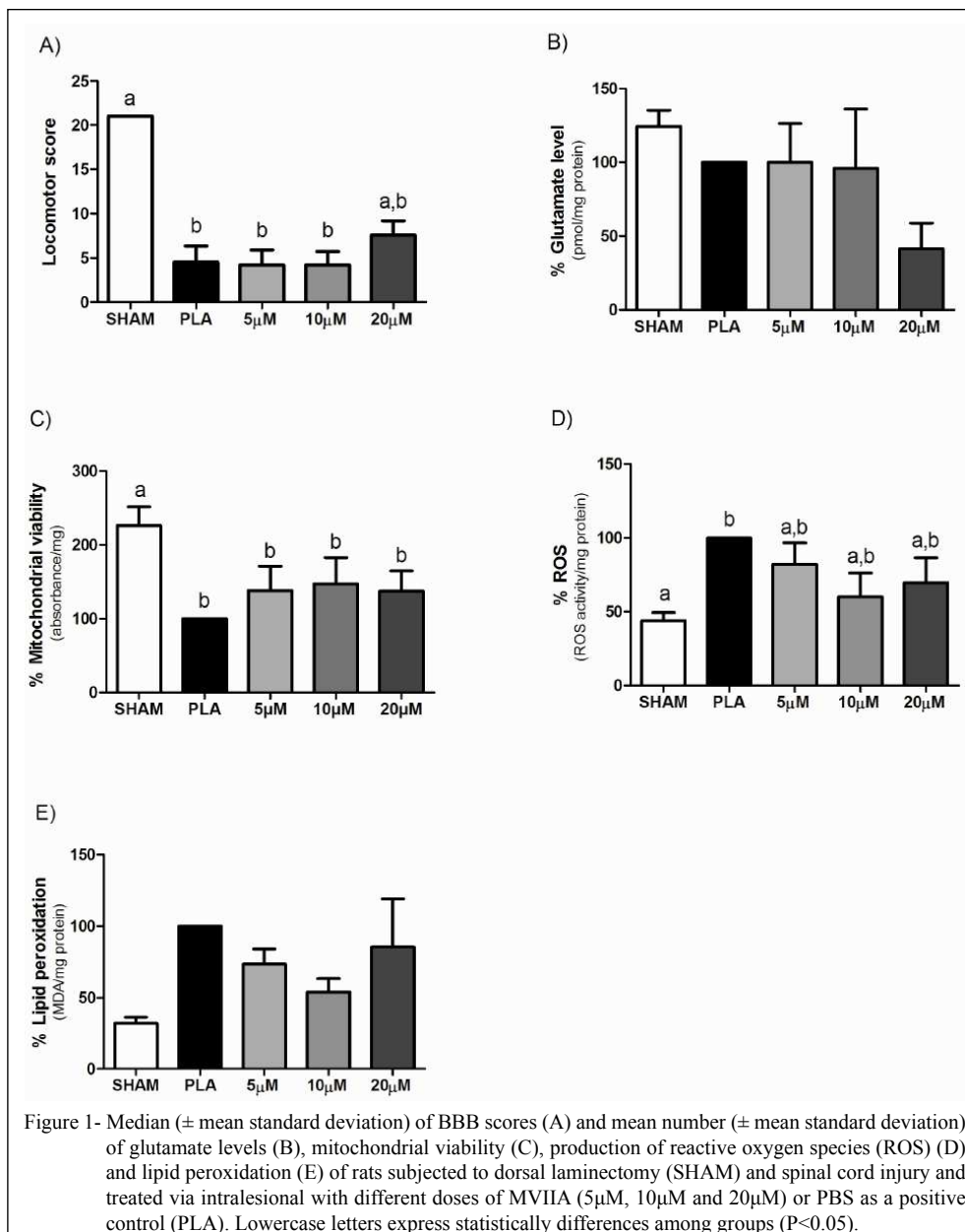
## RESULTS AND DISCUSSION

The model of experimental acute spinal cord injury allowed the reproduction of a moderate to severe trauma, easy, inexpensive and standardized. Before surgery, all animals showed clinical neurological parameters within the normal

range, which was represented by score 21 on the scale of BASSO et al. (1996). Twenty-four hours after surgery, there was no difference between groups subjected to trauma treated with IL route (PLA,  $4 \pm 4.5$ ,  $5 \mu\text{M}$ ,  $2 \pm 4.2$ ;  $10 \mu\text{M}$ ,  $3 \pm 4.2$ ;  $20 \mu\text{M}$ ,  $7$ ,  $6 \pm 3.58$ ) (Figure 1A), which showed standardized SCI.

Due to MVIIA peptidic nature, the cone snail toxins are not orally available and they must be delivered directly into the central nervous system (CNS) (SNUTCH, 2005). Thus, IL route was chosen by allowing the exact site of treatment. Although it is not the most feasible clinically, is an easy, and safe method.

This is the first study that evaluates the neuroprotective effects of  $\omega$ -conotoxin MVIIA *in vivo* in rats subjected to SCI. In this experiment, MVIIA concentrations were chosen from 5 to  $20 \mu\text{M}$  of MVIIA due to the variability of doses reported in the literature, mostly antinociceptive studies where concentrations of 3 to  $200 \mu\text{M}$  showed efficacy in blocking the N-type VDCC (SOUZA et al., 2008; HAMA & SAGEN, 2009; SOUZA et al., 2011; SOUZA et al., 2013), with side effects as a limiting factor. In agreement with HAMA & SAGEN (2009), the lowest dose ( $5 \mu\text{M}$ ) had no complications. But



higher concentrations showed dose-dependent side effects as tremor of the body and tail graded in moderate and severe with doses respectively of 10 (80%, 4/5) and 20 $\mu$ M (100%, 5/5).

The time of application of five minutes after the SCI was chosen in order to early block the N-type VDCC and decrease injuries resulting from exaggerated influx of calcium and its deleterious effects (DASURI et al., 2013). In view of the toxin rapid onset action, approximately 22 minutes (BOWERSOX & LUTHER, 1994), it was believed that this moment would inhibit the first increase of calcium in the 45<sup>th</sup> minute and his subsequent elevation (HAPPEL et al., 1981). Moreover, this time was essential to use the IL route, taking advantage of the same surgical procedure of SCI, which avoided another surgery on the same day, greater tissue trauma, inflammation and animal stress. Besides that, as it is the first research with MVIIA in SCI, and the present research initially decided to study the possible beneficial effects of the conotoxin in early application to subsequently permit future evaluation of the therapeutic window and its clinical feasibility.

In this MVIIA application model, the glutamate concentrations in cerebrospinal fluid did not differ 48 hours after SCI, reaching the level of the negative control (SHAM), possibly by the time of evaluation since it is reported normal levels of glutamate in three hours after SCI. Mean ( $\pm$  standard deviation) concentration of glutamate in the cerebrospinal fluid compared to placebo (100) were 124.14 $\pm$ 22.46 (SHAM), 100.1 $\pm$ 58.21 (5 $\mu$ M MVIIA), 95.92 $\pm$ 89.75 (10 $\mu$ M MVIIA) and 52.02 $\pm$ 35.25 (20 $\mu$ M MVIIA) (Figure 1B). Despite the observation of MVIIA in reducing glutamate at early times (SOUZA et al., 2008; GONÇAVES et al., 2011), some authors pointed out that this fact is not the only implicated in toxin neuroprotection (VALENTINO et al., 1993), since high doses are required for regulation of glutamate release in less than 50% when compared to effective inhibitors levels (BOWERSOX et al., unpublished data, 1999 cited by WANG & BOWERSOX, 2000).

Regarding mitochondrial viability, the MVIIA concentrations did not show cell preservation and the trauma groups were significantly lower when compared to SHAM (226.33 $\pm$ 61.89). The test results were 151.69 $\pm$ 80.75 (5 $\mu$ M MVIIA), 161.5 $\pm$ 87.26 (10 $\mu$ M MVIIA) and 150.77 $\pm$ 67.58 (20 $\mu$ M MVIIA) (Figure 1C). Interestingly VALENTINO et al. (1993), in cerebral ischemia model, and VERWEIJ et al. (2000), in traumatic brain injury, observed that MVIIA injected 15 minutes before or within one hour after the injury did

not preserve the mitochondria as applied late with four or six hours after cerebral ischemia (VALENTINO et al., 1993; VERWEIJ et al., 2000). Then, VERWEIJ et al. (2000) suggested that MVIIA would be more efficient during critical periods of calcium.

When comparing the groups treated with MVIIA in 5 $\mu$ M (82.17 $\pm$ 32.44), 10 $\mu$ M (60.05 $\pm$ 36.08) and 20 $\mu$ M (69.85 $\pm$ 37.27) concentrations, it was noted that no dose was capable of reducing ROS production in relation to the PLA (100) (Figure 1D). As the mitochondria releases activators of the apoptosis cascade and further enhances the secondary injuries to SCI, as oxidative damage, and ROS production (PATEL et al., 2010), MVIIA did not reduce ROS possibly due to not preserving the mitochondria, in agreement with previous results, or not decreased the calcium entrance.

It is also known that, among these ROS, decomposition of peroxynitrite form highly cytotoxic free radicals leading to PL (DASURI et al., 2013). In this study, MVIIA applied with five minutes was also not effective in reducing the PL after SCI, differing from the SHAM (32.17 $\pm$ 10.84) and PLA (100) ( $P < 0.05$ ). The MDA mean ( $\pm$  standard deviation) were: 73.51 $\pm$ 23.62, 54.09 $\pm$ 20.97 and 85.48 $\pm$ 75.31 to 5 $\mu$ M, 10 $\mu$ M and 20 $\mu$ M MVIIA when compared to PLA (100) (Figure 1E). The attenuation of ROS production is directly related to the decrease in the PL (DASURI et al., 2013). However, this was not seen with MVIIA treatment, probably because the inhibition of ROS was not enough to reduce PL.

Based on our results, when MVIIA applied early to SCI did not preserve a major source of free radicals, mitochondria, was unable to reduce the production of ROS and their secondary reaction, lipid peroxidation, and did not promote decrease in the levels of glutamate. In this study, MVIIA possibly did not reduce significantly the intracellular calcium entry, enabling large formation of oxidative damages with consequent loss of mitochondrial viability and lack of neuroprotective effects.

## CONCLUSION

The results demonstrate that early administration of MVIIA via IL route did not promote the reduction of the secondary events to SCI in rats.

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## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

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