

Original Article

Omega-conotoxin MVIIIC attenuates neuronal apoptosis *in vitro* and improves significant recovery after spinal cord injury *in vivo* in rats

Karen M Oliveira¹, Mário Sérgio L Lavor², Carla Maria O Silva¹, Fabíola B Fukushima³, Isabel R Rosado¹, Juneo F Silva¹, Bernardo C Martins¹, Laís B Guimarães¹, Marcus Vinícius Gomez⁴, Marília M Melo³, Eliane G Melo¹

¹Departamento de Medicina e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte-Minas Gerais, Brazil; ²Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz, Ilhéus-Bahia, Brazil; ³Universidade Federal do Paraná, Campus de Palotina, Palotina-Paraná, Brazil; ⁴Laboratório Instituto Nacional de Ciência e Tecnologia de Medicina Molecular, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte-Minas Gerais, Brazil

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Abstract: Excessive accumulation of intracellular calcium is the most critical step after spinal cord injury (SCI). Reducing the calcium influx should result in a better recovery from SCI. Calcium channel blockers have been shown a great potential in reducing brain and spinal cord injury. In this study, we first tested the neuroprotective effect of MVIIIC on slices of spinal cord subjected to ischemia evaluating cell death and caspase-3 activation. Thereafter, we evaluated the efficacy of MVIIIC in ameliorating damage following SCI in rats, for the first time *in vivo*. The spinal cord slices subjected a pretreatment with MVIIIC showed a cell protection with a reduction of dead cells in 24.34% and of caspase-3-specific protease activation. In the *in vivo* experiment, Wistar rats were subjected to extradural compression of the spinal cord at the T12 vertebral level using a weigh of 70 g/cm, following intralesional treatment with either placebo or MVIIIC in different doses (15, 30 and 60 pmol) five minutes after injury. Behavioral testing of hindlimb function was done using the Basso Beattie Bresnahan locomotor rating scale, and revealed significant recovery with 15 pmol (G15) compared to other trauma groups. Also, histological bladder structural revealed significant outcome in G15, with no morphological alterations, and anti-NeuN and TUNEL staining showed that G15 provided neuron preservation and indicated that this group had fewer neuron cell death, similar to *sham*. These results showed the neuroprotective effects of MVIIIC in *in vitro* and *in vivo* model of SCI with neuronal integrity, bladder and behavioral improvements.

Keywords: *In vitro*, cell death, bladder, clinical recovery, neuron preservation, conotoxin

Introduction

Spinal cord injury (SCI) is a leading cause of permanent disability in young adults. It is estimated that the worldwide annual incidence of SCI is 15-40 cases per million of population [1-3].

At the time of trauma, the primary lesion leads to the disruption of axons, neurons, and neuroglia cell bodies, resulting in the interruption of nerve impulses. Further, start the secondary neurodegenerative events, which worsen the initial injury. Excessive accumulation of intracellular calcium is a common phenomenon

after SCI and is the most critical step in ionic dysregulation that generate axonal injury and eventual apoptosis or necrosis via an increase in the activation of cellular enzymes, mitochondrial damage, acidosis, and production of free radicals [4-8].

The neurological deficits from the SCI result in direct interruption of neuronal pathways immediately after the mechanical event. The loss of motor and sensory function distal to the point of SCI leads to multiple health problems such as rashes, urinary retention, urinary tract infection, pressure sores, and respiratory and cardiac dysfunction, reducing quality of life and life

expectancy [2, 9-13]. Bladder dysfunction has consistently been ranked as one of the top concerns among paraplegics and quadriplegics, usually of higher importance than the loss of locomotion [14-17].

The success of SCI treatment depends on how efficiently the secondary injury mechanisms can be altered [7]. Calcium channel blockers (CCB) have been shown to have great potential in reducing brain and spinal cord injury, by preventing the intense influx of this ion and, consequently, the secondary injury progression [18-22]. A wide variety of natural CCB have been identified with neuroactive or neuroprotective peptides derived from different venomous species such as toxins from cone snail of the genus *Conus*, also called conotoxins [23-27].

Omega-conotoxin MVIIC (MVIIC) is a member of the CCB toxin family, constituted by 26 amino-acids [28]. It inhibits, selectively, types N ($Ca_v2.1$) and P/Q ($Ca_v2.2$) voltage-dependent calcium channels (VDCC) that are essential in the release of neurotransmitters related to the development of secondary injury [29-32]. In recent years, it has been shown that the calcium influx and glutamate release [33, 34] can be significantly reduced through blockade of VDCC in several *in vitro* models of brain ischemia [30, 35-37] and spinal injury [38-40]. Thus, these data suggest a potential role of MVIIC in preventing secondary injury. The aim of this study was to determine the neuroprotective effect of MVIIC following SCI through evaluation of neuronal and bladder preservation, neuronal cell death, and clinical recovery.

Materials and methods

In vitro experiment

Rat spinal cord preparation

The experiment was performed in fifteen male Wistar rats, weighting 200 to 250 g, in accordance with the guidelines of Federal University of Minas Gerais Animal Care and Use Committee. Spinal cords were carefully dissected out after guillotine decapitation by hydraulically extrusion as previously described [41]. Briefly, the tip of a 20 ml syringe with 10 ml of cold (4°C) artificial cerebrospinal fluid (ACSF) ($NaCl$ 127 mmol/L; KCl 2.0 mmol/L; $NaHCO_3$ 26.0 mmol/L; $MgSO_4$ 2.0 mmol/L; $CaCl_2$ 2.0 mmol/L; KH_2PO_4 1.2 mmol/L; HEPES: 13.0 mmol/L; pH 7.4) and glucose (10 mM) (ACSF-

10) was attached to the caudal opening of the vertebral canal. The spinal cord was extruded from the end of the vertebral canal by applying pressure on the syringe. The isolated spinal cord was then incubated in a standard ACSF at 4°C and the pia mater and nerve roots were removed. The lumbosacral spinal cord was quickly cut approximately to 400 μm thick transverse slices by cutter tissues (McIlwain Tissue Chopper, Brinkman Instruments, UK), and reported to the chamber perfusion (Brandel Suprafusion System SF-12, Gaithersburg, MD, USA) for subsequent procedures.

Perfusion chamber procedures and treatment

The slices were placed into wheels containing ACSF completely submerged and superfused at a rate of 0.5 ml/min with ACSF, that was saturated with 95% O_2 and 5% CO_2 for 90 minutes to recovery of mechanical dissection trauma. After preincubation in ACSF, spinal cord slices were subject to the following conditions: SHAM group: perfusion in ACSF-10 and 95% O_2 e 5% CO_2 throughout the experiment; ISCHEMIA group: the ischemia was induced by ACSF with oxygen/glucose (4 mM) deprivation (ACSF-4) and continuously gassed with 95% N_2 and 5% CO_2 for 45 minutes; MVIIC group: the pretreatment was done with ACSF-10 and 95% O_2 e 5% CO_2 and MVIIC (1 μM) for 30 minutes followed by ACSF with oxygen/glucose deprivation and continuously gassed with 95% N_2 and 5% CO_2 for 45 minutes. Consecutively, the *in vitro* reoxygenation was induced by the incubation of spinal cord slices in the ACSF containing glucose and continuously saturated with 95% O_2 and 5% CO_2 for 4 hours.

Quantification of dead cells

Using Apotome microscope, dead cell counting was performed based on the membrane-impermeant viability indicator ethidium homodimer-1 (EthH). This high-affinity nucleic-acid stain binds DNA of dead cells and emits red fluorescence. Spinal cord slices were incubated for 30 min at room temperature in 2 mM EthH-1 and ACSF (3 μL EthH : 1 ml ACSF). After washing the slices in ACSF solution for 15 minutes, it was examined under a fluorescent microscope, evaluating the spinal cord ventral horn, and cells were counted using the counting tool of Image J (Image Processing and analysis in JAVA, USA). Data were expressed as dead cells per field \pm SEM (n = 5).

Real time quantitative PCR (qRT-PCR)

The relative quantification of the caspase-3 gene expression in the spinal cord slices were determined as mean \pm SEM for each experimental group (n = 3). The spinal cord slices were flash-frozen in liquid nitrogen, and the total mRNA was extracted by adding the Trizol reagent (Gibco) according to the manufacturer's instructions. One microgram of RNA was subjected to cDNA synthesis by using a SuperScript III Platinum Two-Step qPCR kit with SYBR Green (Invitrogen). The qRT-PCR reactions were conducted in a Smart Cycler II thermocycler (Cepheid Inc.). The one step qRT-PCR amplification started with reverse transcription for 120 s at 50°C, followed by PCR with the following parameters: 45 cycles of 15 s at 95°C for denaturation, 60 s at 60°C for annealing, 60 s at 75°C for extension and 10 minutes at 75°C to the end of the reaction. At the end of each run, fluorescence data were analyzed to obtain cycle threshold (CT) values. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where the values from the samples were averaged and calibrated in relation to β -actin CT values. The primer for the rat genes was as follows: sense 5'-TGGAGGAGGCTGACCGCAA-3', antisense 5'-CTCTGTACCTCGGCAGGCCTGAAT-3' for caspase-3; and sense 5'-GCGTCCACCCGCGAGTACAA-3', antisense 5'-ACATGCCGGAGCCGTTGTCG-3' for β -actin.

In vivo experiment

Housing and surgery

Thirty-six adult, male Wistar rats weighting 400 to 450 g were randomly distributed into six 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. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Minas Gerais (CETEA/UFMG, protocol n° 075/10). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Animals were premedicated with tramadol chloride (4 mg/kg, subcutaneously), and anesthesia was induced and maintained with isoflurane

in a non-rebreathing circuit, through a face-mask. The animals were positioned in a stereotactic apparatus, received prophylactic antibiotic with cephalotin (60 mg/kg, 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 bluntly 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 [16, 21, 42] for five minutes, using a weight of 70 g/cm. Five minutes later, an intralaminar injection was performed according to the experimental group. The incision was closed in two layers and the animals were allowed to recover from anesthesia in a warmed (37°C) box.

Post-operative care procedures involved manual expression of the bladder, three times a day, tramadol chloride (2 mg/kg, orally, every 8 hours) for three days, and cephalexin (30 mg/kg, orally, twice daily) for five days.

Pharmacological treatment of animals

Five minutes after injury, an intralaminar application of 2 μ L of treatment was delivered into the injury center using a Hamilton microsyringe, as previously described [43]. The animals were randomly distributed into six groups, with six rats each, according to the treatment protocol: sham-operated (*sham*), placebo treatment with water for injection (PLA), 15 pmol MVIIC (G15), 30 pmol MVIIC (G30), and 60 pmol MVIIC (G60). For eight days, experiments focused on behavioral recovery. All animals were euthanized at Day 8 following SCI. Behavioral and histopathological evaluations were carried out by investigators who were blind to the experimental conditions.

Open field evaluation

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 every day for seven days after trauma. The hindlimb function

Table 1. Bladder scores according to the lesion histological pattern

Scores	Histological pattern	
	Intensity	Extension
1	Mild	Focal
2	Mild	Multifocal
3	Mild	Diffuse
4	Moderate	Focal
5	Moderate	Multifocal
6	Moderate	Diffuse
7	Severe	Focal
8	Severe	Multifocal
9	Severe	Diffuse

was assessed using the Basso Beattie Bresnahan (BBB) locomotor rating scale [44]. Test sessions were four minutes in duration and rats were tested once a day.

Histological analysis of tissue injury

On Day 8 after surgery, the rats were deeply anesthetized with an overdose of sodium thio-pental (100 mg/kg), intraperitoneally. The animals were perfused with 300 ml 0.9% sodium chloride saline followed by 300 ml of 10% phosphate-buffered formalin (pH 7.4). Following perfusion, the spinal segment between T3 and L3 and the bladder were removed, placed overnight in 10% phosphate-buffered formalin (pH 7.4) and embedded in paraffin. Spinal cord transverse sections (4 µm) were obtained rostral to the lesion epicenter, in a total of three blade spacing 100 µm between them.

Hematoxylin and eosin-bladder evaluation: Histological analysis was also carried out on paraffin-embedded bladder samples. Briefly, 4 µm thick longitudinal sections were stained with hematoxylin and eosin. Lesion areas from sections were classified in nine grades, according to the histological pattern of intensity (mild, moderate, and severe) and extension (focal, multifocal, and diffuse) of the lesion (Table 1).

Anti-NeuN staining and positive neurons counting: Immunohistochemistry using the monoclonal antibody anti-NeuN (Chemicon, cat# MAB377. Temecula, CA, USA) was performed to evaluate neuronal viability. Biotin-streptavidin peroxidase (Laboratory Vision Corp., Fremont, CA, USA) and antigenic recovery techniques with a retrieval solution (sodium

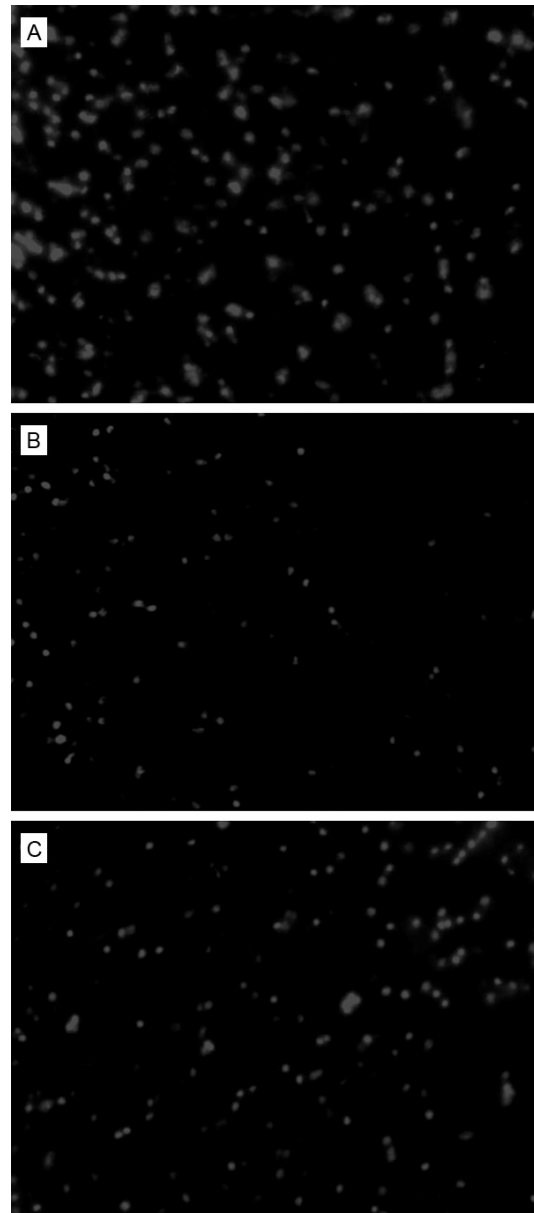


Figure 1. Fluorescence photomicrographs of lumbar-sacral spinal cord ventral horns. The photomicrographs shows fluorescent dead cells in ischemic (A), control (B) and MVIIC pretreatment + ischemia group (C).

citrate 0.5% pH 6.0) were employed. Histological sections were incubated overnight in a humid chamber with primary antibodies (diluted 1:1000) and followed by 30 min during the steps of blocking endogenous peroxidase, blocking serum (DAKO), and streptavidin-biotin-peroxidase. Incubation with the secondary antibody was performed for 45 min. The chromogen utilized was diaminobenzidine (DAB

MVIIC in spinal cord injury

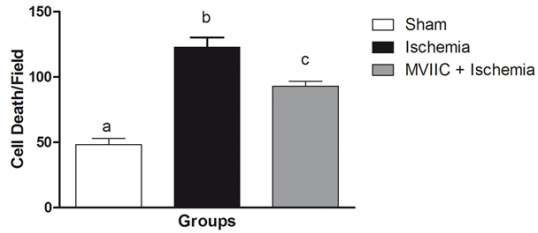


Figure 2. Neuroprotective effect of MVIIC in ischemic slices of rat spinal cord. The results express the mean \pm SEM of cells stained with ethidium homodimer per field in spinal cord slice of negative control (SHAM), ischemic group, and pretreatment with MVIIC + ischemia. Lowercase letters express statistically differences among groups after seven days of spinal cord injury (SNK test; $P < 0.01$).

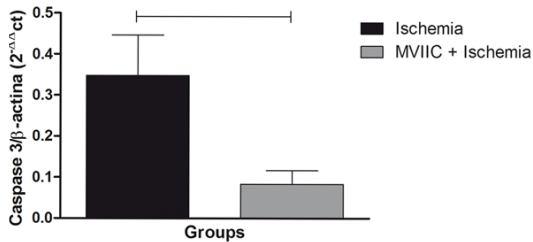


Figure 3. Reduced apoptosis activation by MVIIC following ischemic spinal cord injury. Mean number of caspase-3 expression in spinal cord slice of ischemic group, and pretreatment with MVIIC + ischemia. Lowercase letters express statistically differences among groups after seven days of spinal cord injury (Student *t* test; $P < 0.05$).

substrate system) for 27 seconds. Slides were counterstained with Harris haematoxylin. A negative control was obtained by replacing the primary antibodies with PBS.

The mean number of NeuN-positive neurons was determined in 10 fields within the grey matter with the aid of a 121-point graticule attached to the microscope 40 x objective.

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay: DNA fragmentation in the grey and white matter was evaluated by TUNEL assay (TdT mediated dUTP nick endlabeling), using an in situ apoptosis detection kit (TdT-FragEL® DNA Fragmentation Detection Kit; Calbiochem, San Diego, CA, USA). Antigenic recovery was performed with proteinase K (20 g/ml PBS) for 15 min at room temperature. The slides were incubated in a humid chamber at 37°C with TdT (TdT Equilibration Buffer and TdT Labeling Reaction

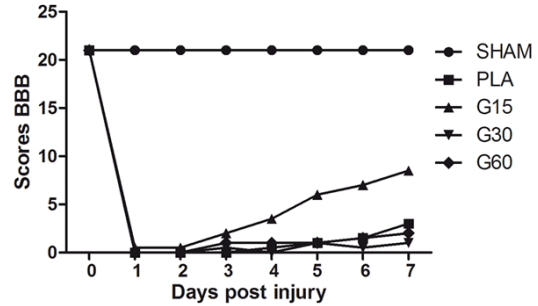


Figure 4. MVIIC improves behavioral scores following SCI. Locomotor evaluation results using Basso Beattie Bresnahan scores during the seven-day evaluation period in rats subjected to compressive spinal cord injury and treated with placebo (PLA, positive control) or ω -conotoxin MVIIC (G15, 15 pmol MVIIC; G30, 30 pmol MVIIC; G60, 60 pmol MVIIC). Lowercase letters express statistically differences among groups, after seven days of spinal cord injury (Mann-Whitney test; $P < 0.05$).

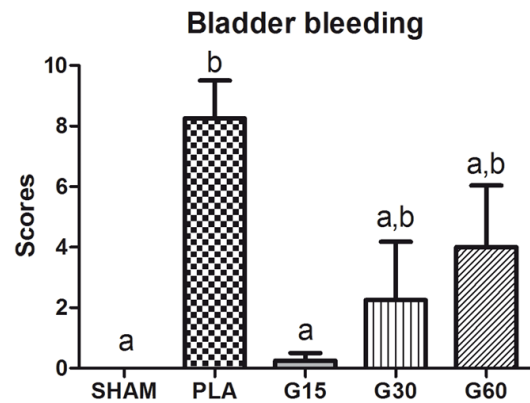


Figure 5. Bladder protection by MVIIC following spinal cord injury. Median \pm SEM scores for bladder bleeding in rats submitted to sham surgery (SHAM) or compressive spinal cord injury and treatment with placebo (PLA, positive control) or ω -conotoxin MVIIC (G15, 15 pmol MVIIC; G30, 30 pmol MVIIC; G60, 60 pmol MVIIC). Lowercase letters express statistically differences among groups, after seven days of spinal cord injury (Kruskal-Wallis test and Dunn's post hoc test; $P < 0.05$).

Mixture) for 1 h at room temperature for the steps of blocking endogenous peroxidase and streptavidin. Endogenous peroxidase activity was blocked with 3% H₂O₂ methanol PBS at room temperature for 5 min. Sections were incubated in TdT buffer solution for 10 min, and then incubated with a mixture containing TdT (TdT Equilibration Buffer and TdT Labeling Reaction Mixture) at 37°C for 1 h. The chromogen DAB was utilized and incubated for 3-6

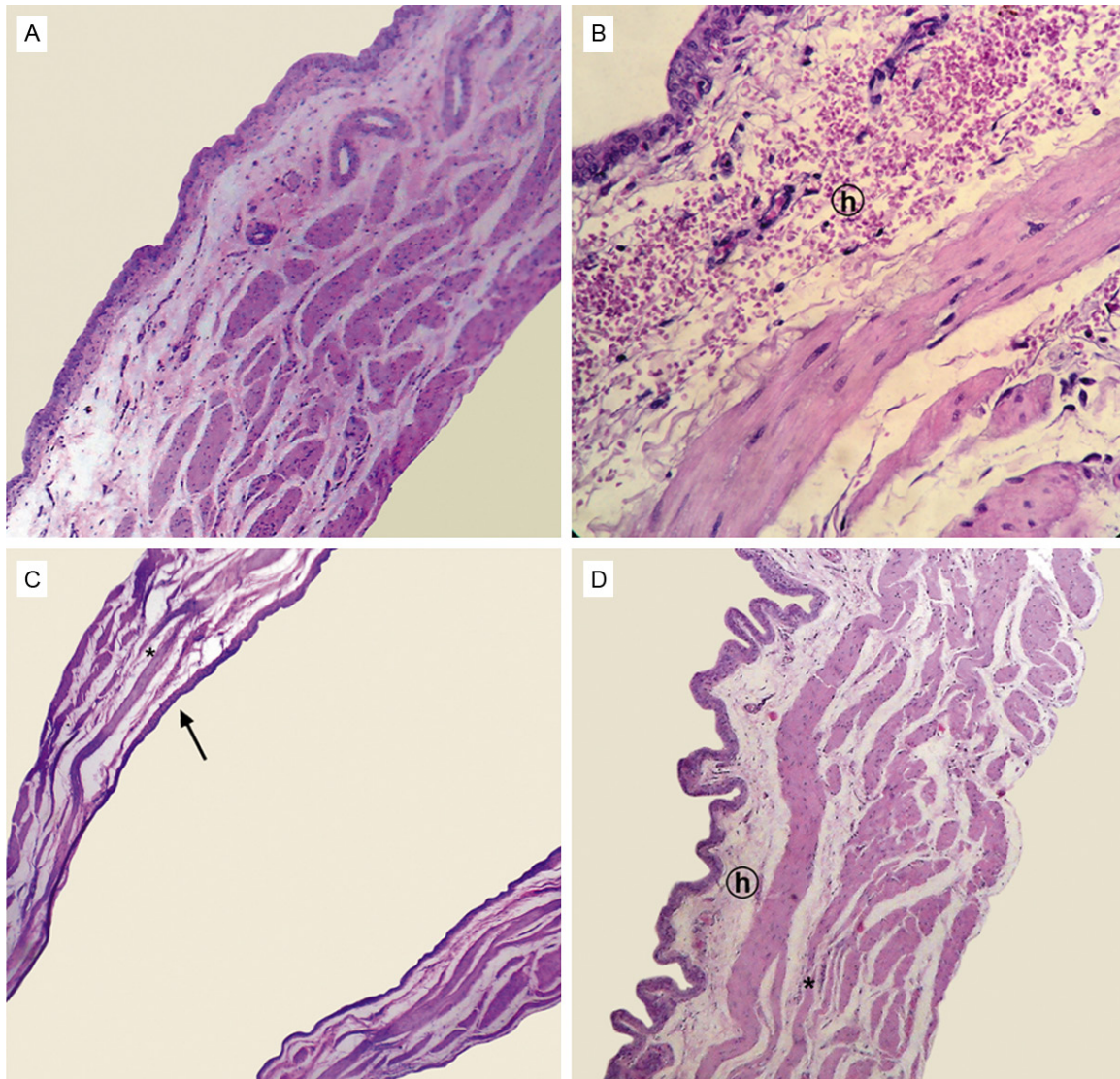


Figure 6. Light microscopy sections of urinary bladder of Wistar rats stained with hematoxylin-eosin. (A) Normal urinary bladder of an animal from SHAM (non-injured) - 101.4x, (B) Multifocal areas of hemorrhage (h) in a injured group placebo-treated animal - 140x, (C) Uroepithelium consisting of only one or two cells layers (arrow) and bladder muscularis overdilation (asterisk) in a placebo-treated animal - 48.6x, (D) Urinary bladder in an injured animal MVIIC-treated (15 pmol MVIIC) showing less hemorrhage and distension - 97.1x.

min. Sections were counterstained with methyl green. The negative control was obtained by replacing TdT with Tris-buffered saline (TBS).

The mean number of TUNEL positive cells (TPC) was determined in 15 fields within the grey matter with the aid of a 121-point graticule attached to the microscope 40 x objective.

Statistical analysis

All data collected were analyzed using Prism 5 for Windows (GraphPad Software, La Jolla, CA,

USA). Data from BBB locomotor rating scale between groups were evaluated using Mann-Whitney test and between days by Friedman test. The bladder lesion was compared by Kruskal-Wallis test and Dunn's post hoc test. Data from number of NeuN-positive neurons did not follow a normal distribution and were evaluated by Kruskal-Wallis test. Others 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.

MVIIC in spinal cord injury

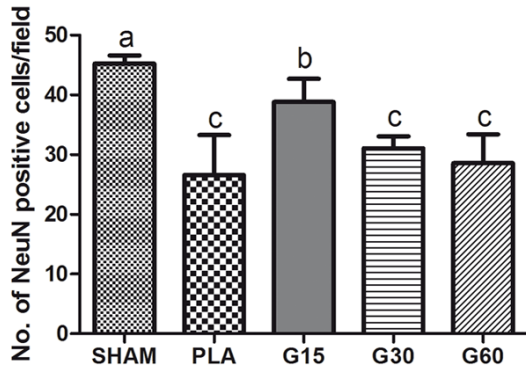


Figure 7. Neuroprotective effect of MVIIC after spinal cord injury in rats. Mean number of NeuN positive neuronal cells per field in the cranial spinal cord segment of rats submitted to sham surgery (SHAM) or compressive spinal cord injury and treatment with placebo (PLA, positive control) or ω -conotoxin MVIIC (G15, 15 pmol MVIIC; G30, 30 pmol MVIIC; G60, 60 pmol MVIIC). Lowercase letters express statistically differences among groups after seven days of spinal cord injury (Kruskal-Wallis test; $P < 0.05$).

Results

In vitro experiment

MVIIC confers cell preservation following ischemic insult

The neuroprotective effect of MVIIC on slices of spinal cord subjected to ischemia was tested against cell death. The spinal cord slices subjected a pretreatment with MVIIC showed a cell protection with a reduction of dead cells in 24.34%. The number of dead cell was significantly lower in MVIIC (median \pm SD: 93.03 ± 8.12) than ischemic group (122.97 ± 16.4) ($P < 0.01$) (Figures 1 and 2).

MVIIC reduces apoptosis activation following ischemic insult

To determine whether the reduction of neuronal cells death is accompanied by less activation of apoptosis-specific proteases, we measured the caspase-3 expression. These data demonstrate that caspase-3-specific protease is less activated in MVIIC (mean \pm SD: 0.083 ± 0.058) presence than ischemia (0.347 ± 0.171) group ($P < 0.05$) (Figure 3).

In vivo experiment

The model of experimental acute spinal cord injury allowed the reproduction of a moderate

to severe trauma, easy, inexpensive and standardized. The weight of 70 g directly on the spinal dura in the region of T12 for five minutes caused severe paraplegia of hindlimbs in all animals and urinary retention. Intralesional administration of MVIIC was chosen in the present study due to the reduced risk of systemic side effects and enhanced spinal effect.

MVIIC improves behavioral scores following SCI

Repeated measures analysis of hindlimbs locomotor function revealed significant recovery of hindlimb function with 15 pmol MVIIC compared to 30 and 60 pmol MVIIC at Day 4 ($P = 0.026$) and placebo group at Day 5 ($P = 0.0432$) (Figure 4). Comparing the days of evaluation, G15 recovered faster than G30 and G60, with significant different beginning on Day 6. G15 achieved a final average score of 8.5 (indicative of smooth movements, weight support, and occasionally plantar stepping) at Day 7 post-injury compared to 3, 1, and 2 (indicative of slight movements of joints) for PLA, G30, and G60, respectively.

MVIIC confers bladder protection following SCI

Histological evaluation of the bladders revealed hemorrhagic foci in those subjected to trauma and treated with placebo, 30, and 60 pmol MVIIC, but no morphological alterations were observed in the bladders of sham and G15 groups (Figure 5). The categorization of these foci in the bladder showed that animals receiving placebo had significantly more bleeding compared to sham and G15 groups ($P < 0.05$) (Figure 6).

MVIIC confers neuroprotection in SCI

Anti-NeuN staining and positive neurons counting: Positive neurons invariably had preserved morphological characteristics. The number of NeuN-positive cells (NPC) was significantly higher in G15 (38.8 ± 3.90) when compared to PLA (26.58 ± 6.71), G30 (31.07 ± 2), and G60 (28.58 ± 4.83) ($P < 0.05$) (Figures 7 and 8), although statistically lower than sham group (43.87 ± 3.52).

TUNEL assay: The number of TPC in white and grey matter cranially the lesion epicenter tended to be lower in G15 (1.07 ± 0.76) than trauma groups and became similar to sham group

MVIIC in spinal cord injury

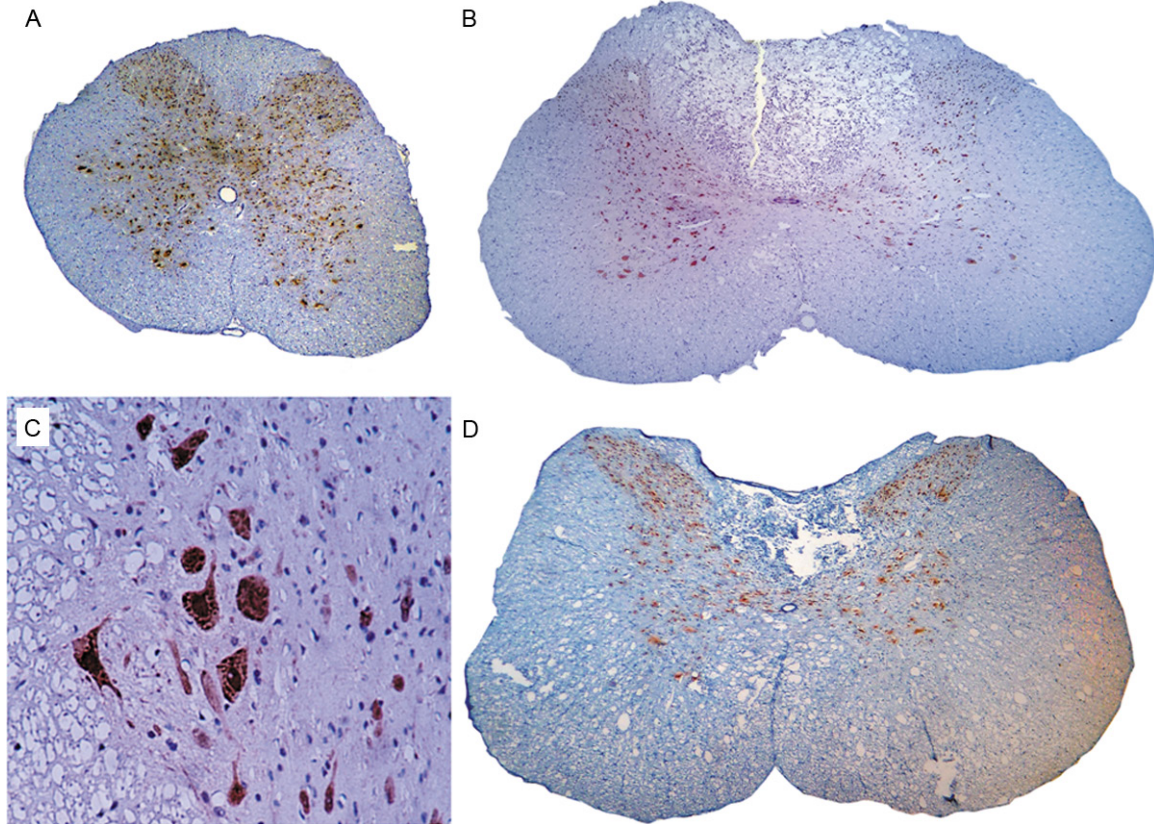


Figure 8. Light microscopy of spinal cord sections of Wistar rats stained with anti-NeuN. (A) Nuclei staining of intact neuronal cell bodies (NeuN-positive) in the gray matter in sham-non injured animal - 22.63x, (B) Mild staining of NeuN-positive cells in placebo-injured animal - 36.57x, (C) Detail of NeuN-positive cell neurons - 118.57x, (D) Moderate staining of NeuN-positive cells in G15 (15 pmol MVIIC) animal - 35.71x.

(0.16 ± 0.11) ($P > 0.05$), but did not differ from PLA (1.61 ± 0.53), G30 (1.93 ± 0.58), and G60 (1.88 ± 1.42) (Figure 9).

Discussion

Activation of VDCC is one of the most important routes of calcium entrance and represents a key step in the regulation of cellular processes [30] and excitability, participating actively in the acute neurodegenerative process [22, 45, 46]. The secondary lesions initiate minutes after mechanical trauma. These events result from excessive release and inadequate reuptake of glutamate [40, 47, 48], leading to a prolonged excitatory synaptic transmission [9, 49] with excessive Na^+ and Ca^{2+} influx by activation of ionotropic, metabotropic and VDCC receptors, during membrane depolarization [22, 50-52].

The fact that damage continues to develop over time during the days and weeks following acute SCI provides an opportunity to intervene.

Neuroprotective strategies aimed at preventing damage arising from secondary injury processes provide some hope for tissue sparing and improved functional outcome. It is speculated that blocking VDCC protects the structural integrity of oligodendrocytes and astrocytes due to the presence of N-type channels in their membranes [23-26, 45, 53]. And can thus, in long term evaluation, be beneficial to neurological improvement after SCI [17-19, 54].

It has been demonstrated in models of cerebral ischemia that ω -conotoxins exert neuroprotective effects by antagonizing VDCC and thereby inhibiting excessive release of neurotransmitters [23, 24, 31, 32, 55]. Specifically, MVIIC selectively inhibits VDCC type N, P, and Q that are essential for the release of neurotransmitters associated with the development of secondary injury [29-31, 34]. Therefore, our *in vitro* results showed the effects of MVIIC on caspase-3 expression and cell death reduction that can be explained because the blockade of calcium channel diminishes the calcium influx

MVIIC in spinal cord injury

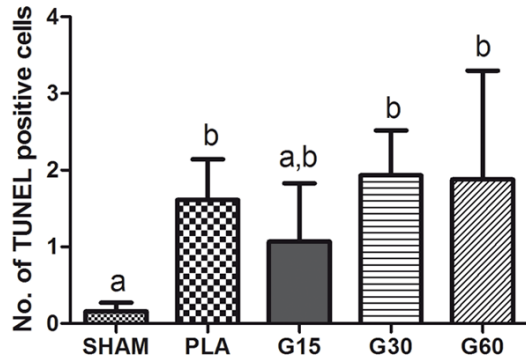


Figure 9. Quantification of apoptotic cells after spinal cord injury. Mean number of TUNEL positive cells in the cranial spinal cord segment of rats submitted to sham surgery (SHAM) or compressive spinal cord injury and treatment with placebo (PLA, positive control) or ω -conotoxin MVIIC (G15, 15 pmol MVIIC; G30, 30 pmol MVIIC; G60, 60 pmol MVIIC). Lowercase letters express statistically differences among groups after seven days of spinal cord injury (Student-Newman-Keuls test; $P < 0.05$).

and their deleterious effects [6, 51, 56, 57]. Calcium overload can trigger a range of calcium-dependent processes that will lethally alter the metabolism of remaining cells [58, 59] as activation of caspases that induces cell apoptosis [7, 8, 60, 61]. Thereby, MVIIC provides protection of spinal cord slices subjected to ischemia.

Given these characteristics, it was postulated that MVIIC administered immediately after SCI, can exert neuroprotective effects, leading to neurological improvement. This is the first study that evaluates the neuroprotective effects of ω -conotoxin MVIIC *in vivo* in rats subjected to SCI. In this experiment, MVIIC doses were chosen based on [62] who showed that 100 pmol had deleterious effects when applied intracerebroventricularly *in vivo*.

In this experiment, we found that 15 pmol MVIIC promoted significant recovery of rats from SCI. Until three days following SCI all traumatized rats exhibited severe deficits in hindlimb function and locomotion using forelimbs and dragging hindlimbs. The only observed hindlimb movements consisted of slight-extensive movements of the hindlimb joints, usually hip and knee, with no evidence of weight support. While 15 pmol MVIIC group showed some improvement throughout the duration of the study, recovery in the other trauma groups (PLA, G30 and G60) was limited. In

contrast, 15 pmol MVIIC treated rats showed continual and significant improvements over the seven-day recovery period. Seven days post-injury, the median score for BBB test was 8.5 in G15, with the majority of rats exhibiting scores > 8 . In these animals, consistent weight supported plantar stepping was observed, and some showed evidence of forelimb-hindlimb coordination.

Since the functional recovery of 15 pmol MVIIC group differed from other doses of MVIIC and placebo from the earliest time point studied, respectively, four and five days, the mechanism of 15 pmol of MVIIC is likely to be through neuroprotection. As the other doses were higher than 15 pmol, it is possible that they blocked excessively the calcium channels, and could be deleterious to the cell as calcium regulates many important cellular neural processes, including neurotransmitter release, gene transcription, and cell proliferation [23, 34, 63]. Intracellular calcium concentration is controlled by the balance of signals that determine their entry [51, 64, 65].

In this study, 15 pmol MVIIC provided neuron preservation, identified by immunostaining of the neuronal marker NeuN. High doses of MVIIC did not improve clinical recovery and neuron protection possibly due to excessive blockade of calcium channels, and consequently, negative side effects, since calcium is essential in various cellular processes [51, 63, 65]. NeuN immunoreactivity may decrease in several pathological conditions that affect neuronal viability, such as ischemia, hypoxia, and trauma [66-68]. It can be argued that the neuronal preservation inferred from the NPC data contributed to the improved hindlimb function seen in the G15.

The TUNEL technique allowed the detection of DNA fragmentation of neurons and glial cells [60, 69]. Our findings indicated that neuronal cell death could be detected eight days following traumatic SCI, and interestingly that animals treated with 15 pmol MVIIC had fewer TPC, similar to *sham*, although not different from the other trauma groups. In addition, the number of anti-NeuN immunoreactive neurons was inversely proportional to the number of TUNEL positive cells in G15, indicating the neuroprotective effect of 15 pmol MVIIC.

Moreover, the results of the current study show that MVIIC has also protective effects on spinal cord compression-induced urinary bladder injury. It is known that SCI results in severe functional disturbances of the lower urinary tract [16, 17, 70, 71]. At necropsy, the bladder was distended in most animals subjected to trauma. According to [72], loss of bladder voluntary control, distension, increased bladder pressure, and hemorrhagic cystitis are expected in patients with injuries cranial to the lumbar region. Furthermore, it was observed in all injured animals, epithelial desquamation, as evidenced by the reduced number of layers, representing cell death due to distension and vascular occlusion [70-73]. Categorization of hemorrhagic foci in the bladder showed that animals receiving placebo had significantly more bleeding when compared to *sham* and G15 ($p < 0.05$). The results showed that the 15 pmol MVIIC was able to reduce drastically the hemorrhagic process, resulting in lower intensity of bladder lesions, therefore, suggest a protective action of the drug on that tissue. It is worth noting that, to rule out the possibility that pressure from the manual urine expression was the source of hematuria, in this study *sham* animals also experienced bladder expression, as performed by [21] and [73].

Thereby, clinical recovery, bladder protection, cell death decrease, and neuron preservation confirm, for the first time *in vivo*, the potential neuroprotective role of 15 pmol MVIIC after SCI in rats. There is evidence that MVIIC has a broad spectrum for blocking N, P, and Q types calcium channels [28, 35, 36, 74, 75] and that N-type channel blockade is potent, but of short duration and readily reversible, while P and Q are potent and slowly reversible. Studies in cerebral [30, 35, 36, 57] and spinal cord [38, 39] ischemia demonstrated *in vitro* that the MVIIC significantly reduced calcium influx through types N, P, and Q VDCC, significantly attenuating the release of glutamate [33, 34]. It is tempting to speculate that 15 pmol MVIIC decreases calcium influx and excessive glutamate release and inhibits secondary mechanisms of injury. MVIIC was only used as a research tool claiming to be lethal to mammalian according [76]. Although deleterious effects are frequently reported, including generalized tremors, walking in a circle, muscle weakness or death [62], these did not observe

in the present study, even with higher doses. The effects of MVIIC on locomotor deficits (flaccid paralysis of the hindlimbs or decreased withdrawal tail response) were not evaluated, since these signals are similar to the ones caused by thoracolumbar spinal cord injury. Besides that, the clinical, hematological, biochemical, and histopathological evaluation revealed no significant abnormalities in all groups, in a previous experiment, except for degenerative changes in kidneys at a dose of 120 pmol [77].

In summary, we demonstrate, for the first time *in vivo*, that MVIIC in a rat model of SCI significantly preserves neuronal integrity, prevents bladder bleeding and leads to behavioral improvements. The results confirmed our hypothesis that MVIIC has neuroprotective effects, and that could be possible to use after spinal cord injury.

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Disclosure of conflict of interest

None.

Address correspondence to: Karen M Oliveira, Departamento de Medicina e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627, Pampulha, Belo Horizonte-Minas Gerais, 30123-970, Brazil. Tel: (55) 31 34092266. E-mail: kamoliveira@yahoo.com.br

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