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Analysis of the influence of inoculation of ischiatic nerve fragments in the presence of astragalosíde ivin the neuroprotection of dorsal root ganglions in animals submitted to the transcription of the spinal cord

Eurico de Souza Costa [1]; Dinalva Brito de Queiroz [2]; Amália Cinthia Meneses do Rêgo [2]; Francisco Irochima Pinheiro [2]; Irami Araújo - Filho [2]; Francisco Vitor Aires Nunes[3] and Fausto Pierdoná Guze[1,2]*

[1] Laboratory of Experimental Neurology, Department of Biomedical Sciences, Health Science Center, State University of Rio Grande do Norte, Mossoró-RN, Brazil.

[2] Postgraduate Program in Biotechnology, Health School, Laureate International Universities - Potiguar University, Natal-RN, Brazil.

[3] Course of Medicine of the Federal Rural University of the Semi-Arid - UFERSA. Mossoró-RN, Brazil.

***Corresponding author:** Fausto Pierdoná Guzen, PhD., Full Professor, Department of Biomedic Sciences, Health Sciences Faculty, State University of Rio Grande do Norte, Zip code: 59607-360, Mossoró, Rio Grande do Norte, Brazil. Tel.: +55-84-33152248; e-mail: faustoguzen@uern.br

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Abstract

Introduction: Injury to the cord spinal is a complex medical disease, associated with a serious and disabling incapacity in life, predisposing to a systemic dysfunction that must lead to a secondary process, with a high risk of death. **Objective**: The objective of this study was to analyze the effects of sciatic nerve fragmentation in the presence of Astragaloside IV (AS-IV)

on the neuroprotection of dorsal root ganglia after the transected spinal cord of the rat. **Methods**: The animals were submitted to a 4 mm long medullary transection at the lower thoracic level, and sciatic nerve fragments plus 10 µl of saline solution (5%) were inoculated in the animals of group 1 and group 2, and fragments of sciatic nerves added 10µl of AS--IV. After 3 months the dorsal root ganglia parallel to the spinal cord region were removed and processed for tissue analysis using hematoxylin/eosin staining. Results: In the group treated with sciatic nerve graft AS-IV, we observed the greatest preservation of the morphological root of the dorsal root ganglia when present in the group lacking AS-IV. **Conclusion**: Sciatic nerve grafting with the addition of AS-IV in the mouse transected marrow promotes neuroprotection in the dorsal root ganglion neurons.

1. Introduction

Medullary Lesion (LM), a problematic event in the public health sphere, because it is one of the most devastating lesions, from an organic, psychological and social point of view, and constitutes a major challenge due to its multiple facets. A problematic event that affects mainly young men of productive age, being 15 to 40 people per 1 million inhabitants each year, with great economic impact (Lim, Tow, 2007; Bezerra; Maia and Guzen, 2016).

As a consequence, this population of patients affected by LM would present secondary complications necessitating a longer contact time with the health services. Among the complications are included: musculoskeletal, respiratory, gastrointestinal and urological disorders, which later lead to new hospitalization and home care processes (Chikuda et al., 2014).

Spinal cord injuries can result from direct injury from penetrating wounds (stabbing, projectiles) or indirect injury as a consequence of vertebral fractures or displacements (Rubin et al., 2006). Trauma is thus a major cause of morbidity and mortality worldwide. In Brazil, there are few studies published on the epidemio-

logy of LM, but it is estimated that more than 10,000 new cases occur per year. Individuals with these conditions may have several dysfunctions that negatively interfere with the functional activities and quality of life of the population, balance deficits, muscle tone and joint flexibility that consecutively interfere with gait (Citero; Mederdrut; Fontes, 2012).

A greater part of the affected population is at full productive age and functional independence, after the event they move to a stage where they need help from third parties, reduce self-esteem and increase the cost of living. This condition is one of the challenges for medicine, due to its high incidence and lack of satisfactory treatment (Cristante, Taricco, Colares, 2010).

The pathophysiology of the nerve injury consists of injuries that are divided into two stages: primary and secondary injury. The first consists of the mechanical damage provided by the trauma mechanism. While the second lesion is a pathological process initiated by the first lesion but, with late clinical presentation, occasioned by the cascade of biochemical events (Botelho et al., 2009).

It is known that healthy nerves have the capacity of stretching and sliding to allow the length necessary for the physiological movement of the limbs to increase, but these movements must be in harmony. The traumatic injury of a nerve causes changes in its mechanical and neurochemical properties, leading to the loss of the accommodation characteristic of the movements, with consequent mechanical deficit. However, if the intensity and duration of the compression promoted by the lesion are small, the nerves recover immediately or shortly after the trauma, but if the pressure is intense and / or the duration is long, the recovery will be prolonged and often partial (Machado and Bigolin, 2010).

The regenerative capacity of the neurons depends very much on their microenvironment, since after the injury of the nerve by crushing or axotomy, the distal stump of the peripheral nerve undergoes Wallerian degeneration. Regeneration still depends on some factors, such as: therapeutic

interventions composed of regenerative transplants, neuroprotective drugs, neurotrophic factors, elimination of inhibitory molecules, muscle stimulation (Houl; Cote, 2013), myelin sheath thickness, myelin sheath thickness, among others Powers et al., 2013).

A proposed alternative is herbal medicines, which have scientific relevance in the treatment of neurological diseases, since they contain multiple compounds and phytochemicals that may have a neuroprotective effect, with a consequent beneficial effect on health in different neuropsychiatric and neurodegenerative disorders (Kumar and Khanum, 2012; Guzen et al., 2016). Among these compounds, we can highlight the astragaloside IV (AS-IV), a triterpenoid saponin present in the root of Astragalus membranaceus, a phytotherapeutic origin of traditional Chinese medicine, which is the target of the present study (Li et al., 2017; Costa et al., 2018).

Considering the clinical and social importance of pathologies that involve the spinal cord in its pathophysiological basis, the relevance of the study lies in the search of the effects of sciatic nerve inoculation supplemented with AS-IV on the neuroprotection of the dorsal root ganglia after spinal cord transection.

2. Material and Methods

2.1. Experimental Draw

To perform the experiment, 19 animals (Wistar rats - Rattus novergicus), young adults aged 45 to 55 days were used. The study complied with the norms for carrying out animal research with all procedures, approved by the Ethics Committee on the Use of Animals of the State University of Rio Grande do Norte (UERN), through an opinion substantiated by number 004/17, according to the international norm that governs the research, based on ethical principles adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL), according to the law n° 11.794, of October 8, 2008.

The animals were randomly divided, three animals submitted to the removal of the ischiatic nerves and 16 animals

receiving injury by complete transection of the spinal cord between the T9-10 levels of the thoracic vertebra. After this the animals were divided into two groups: the first group (G1), with 8 animals, was inoculated fragments of the sciatic nerve added saline solution. The second group (G2) also with 8 animals, was inoculated fragments of the sciatic nerve plus AS-IV. The animals were initially pre-anesthetized with 10% Ketamine Hydrochloride using a dosage of 0.1ml / 100gr and 2% Xylazine with 0.01ml / 100gr (Agener Union, Brazil) intraperitoneally. After the surgery, the animals were maintained and monitored for 12 weeks. After time the animals were euthanized, as will be detailed later.

2.2. Removal of the ischiatic nerve

In the three animals submitted to sciatic nerve extraction, the posterior limb tricotomy and local asepsis were performed with soap, water and 70% alcohol. Subsequently, they were anesthetized with 10% 0.1mL / 100g and Xylazine 2% 0.01ml / 100gr (Agener União, Brazil) and surgical accessions were made in the posterior region of the thigh in the two antimeres, removing the muscular planes, resulting in exposure of the sciatic nerve and promoting bilateral withdrawal. They were then placed in the 100mm culture plates (Corning, USA) with approximately 30ml of Leibovitz's -15 (L-15; Gibco, USA) under aseptic surgical technique, according to a method described by Morrissey et al. (1991). After this process the animals were submitted to euthanasia by cervical dislocation. Next, all excess tissue (muscle, fat and blood vessels) adhered to the nerves was removed under magnification by microsurgical stereoscopic magnifying glass (DF Vasconcelos, Brazil). The perineurium was removed from the nerves under a magnifying glass using microsurgical techniques, and placed on a 100mm plate with 20ml of L-15. The nerves were cut into 4mm pieces with a micro-scalp to obtain the explants (Figure 1).

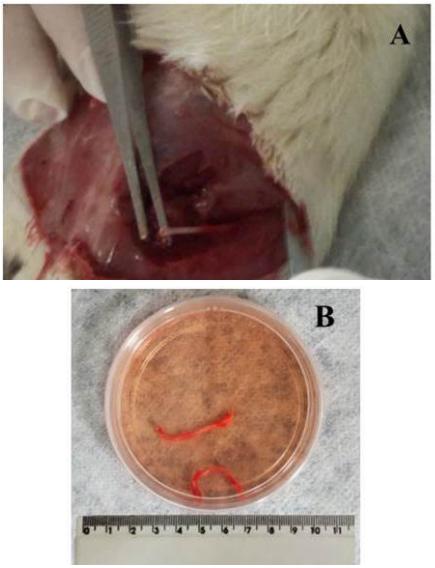


Figure 1. Photograph illustrating the animal being subjected to sciatic nerve removal. Exposure of sciatic nerve (A), Nerves removed and maintained in solution L15 (B).

2.3. Medullar injury

The animals were submitted to tricotomy and local asepsis with 70% alcohol. Initially they were pre-anesthetized with 10% Ketamine Hydrochloride using a dosage of 0.1ml / 100gr and 2% Xylazine with 0.01ml / 100gr (Agener União, Brazil) and maintained throughout the surgical procedure. A longitudinal incision was then made over the lower thoracic region of the animals' backs, removing the deep muscular planes and separating them from the spinal processes of the thoracic vertebrae of the T9-10 levels.

The spinous processes and the posterior arch of these vertebrae were removed by exposing the adjacent spinal segment T10-11. Next, the medullary section was taken along the entire length of its longitudinal axis at two points, with the removal of a segment of approximately 4mm of nerve tissue. Durable 5x5 cm membrane fragments were used for haemostasis. Then, 2 fragments of the sciatic nerve plus 10µl of saline solution (5%) were introduced into each animal in group 1 (G1) and 2 fragments of the sciatic nerve plus 10µl of AS-IV in each animal of group 2 (G2) constituting a final volume of 10µl implanted individually. Finally, suturing of the muscular plane, subcutaneous tissue and skin with absorbent polyglactin wires 4.0 Ethicon was performed.

The animals were placed in cages (n = 4 per cage) with water and feed at will. Bladder emptying was performed daily through

the Credé maneuver (Beattie et al., 1997) until the normal functioning of the urinary bladder was restored. Animals that presented signs of urinary tract infection (dark, fetid or bloody urine) received intramuscular preventive antibiotic therapy (ceftriaxone sodium) (Novartis, 40 mg / kg / day) until observational improvement (Figure 2).

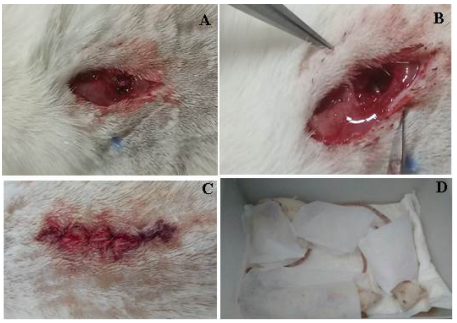


Figure 2. Spinal cord injury procedures. Longitudinal incision at the T9-10 levels of the thoracic vertebrae, removal of the spinal process and visualization of the spinal cord (A). Transection of the spinal cord (B), suturing and identification of the animal (C), post-surgical conditioning of the animals (D).

2.4. Inoculation of astragaloside IV

The AS-IV dry powder extract was obtained from Evidence Soluções Farmacêuticas Ltda®. The concentration used for dilution was 500 µg of AS-IV to 500 µl of double-distilled water (ABD) and stored in the freezer. Then, 10µl aliquot was removed from the freezer and 2.5µl ventral to the graft was added, 2.5µl rostrally to the graft, 2.5µl caudally to the graft and 2.5µl dorsal to the graft totaling 10µl per animal, AS-IV interacts with both areas of the spinal cord injury and the graft.

2.5. Eutanasia process and textile preparation

Twelve weeks after surgery and behavioral analysis, the animals were anesthetized with 10% Ketamine Hydrochloride using a dosage of 0.1ml / 100gr and 2% Xylazine with 0.01ml / 100gr (Agener União, Brazil) intraperitoneally and were euthanized through a transcardiac perfusion of 0.9% saline solution and fixative solution. The fixative solution is a solution consisting of 4% paraformaldehyde diluted in 0.1M phosphate buffer pH 7.4. After decapitation, the tegumen-

tary tissue was removed from the animal's back from the cervical to the lumbar region. Next, a bilateral incision was made in the vertebral column resulting in the removal of spinal and transverse processes going from the cervical to the lumbar region. Carefully removed the graft region, with scalpel, the tissue adhered to the graft was removed.

Segment of the spinal cord were quickly removed. The tissues were postfixed in fixative solution for 90 minutes and washed in a 10% solution of sucrose dissolved in phosphate buffer for 48 hours. The segments of the spinal cords, containing the grafts, were reduced to 1cm, frozen at -80oC for later serial cross sectioning at 30µm. The cuts were performed on a hand-sliding microtome (Leica, Cryocut 1800) with constant temperature maintained. The sections were mounted on gelatinized slides (Fisher Scientific) and processed for labeling.

2.6. Hematoxylin eosin marking

The tissue sections of the spinal cord were stained by the hematoxylin and eosin technique as follows: the blades with the medullary sections referring to the epicenter of the lesion were left in contact with the hematoxylin (Harris method) for 120 seconds and washed in distilled water for 30 seconds and then immersed in eosin solution for 30 seconds. Then the slides passed through an ethyl alcohol battery for 60 seconds, followed by xylol also for 60 seconds. At the end of the battery the slides were rested for 30 minutes for drying and were covered with coverslip with the aid of inclusion medium (Entellan, Merck, Germany) for further analysis under light microscopy.

2.7. Microdensitometric quantitative analysis

Samples were analyzed and the structures were detailed. For this analysis, sections of the epicenter were sampled in the grafts in the spinal cord. The series of stained sections were examined under light microscope (Olympus BX41). The images were made using Motic Images

Plus 2.0 software (Motic®), with the aid of a digital video camera (Nikon DXM1200), which were analyzed, corrected and assembled using Adobe Photoshop CS6.0 software (Adobe®). The results were documented in photomicrographs and graphics format. A 40x magnification immersion lens with a 1.4 aperture was used.

5. Results

Nerve group (2.1 µm2) and the nerve + AS-IV group (2.8 µm2). Considering the injured groups, the group that received sciatic nerve graft with AS-IV added showed higher values of cell body diameter compared to the nerve group (Fig. 3).

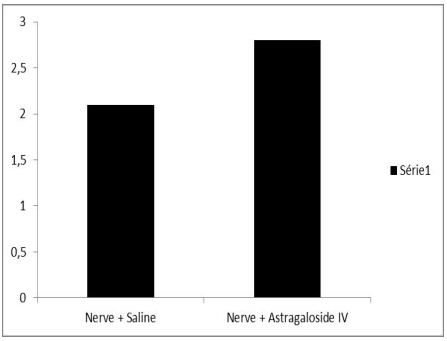


Figura 3. Diameter of cell bodies in the DRG of animals treated with sciatic nerve or fragments of the sciatic nerve with added AS-IV.

Nerve group (7.52 µm2) and the nerve + AS-IV group (8.57 µm2). Considering the injured groups, the group that received sciatic nerve graft with AS-IV added showed higher values of cell body area compared to the nerve group (Fig. 4).

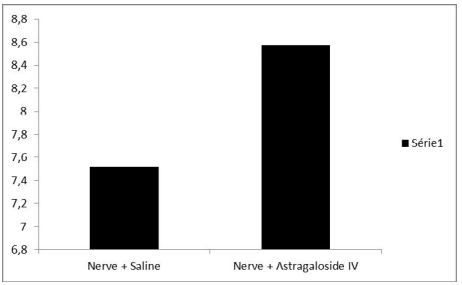
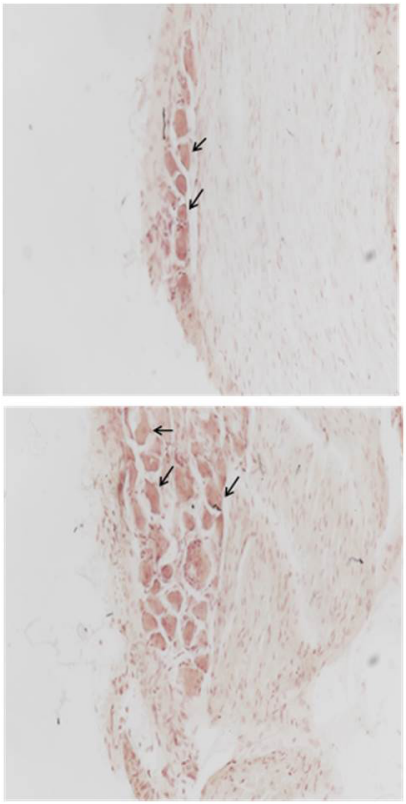


Figura. 4. Area of cell bodies in the DRG of animals treated with sciatic nerve or fragments of the sciatic nerve with added AS-IV.

The group treated with sciatic nerve grafts (A) presented smaller neuronal cell bodies; in addition, a decrease in the area and cellular perimeter was observed when compared to the group treated with sciatic nerve graft with addition of AS-IV (B) (Figure 5).



6. Discussion

As reported, pharmacological therapies are a common option, however, different products developed with active compounds extracted from plants have been evaluated extensively over the years and some have achieved promising results. Currently, there are approximately 350,000 types of natural herbs as sources of drugs in the world and the extracts and compounds alone are beneficial to human health and have shown efficacy in treating pathologies (Wang et al., 2017). In the light of these explanations, among these compounds, we can highlight the AS-IV, a triterpenoid saponin with molecular weight (MW = 784 AD) present in the root of Astragalus membranaceus, a herbal medicine originating in traditional Chinese medicine al., 2017). This small saponin has been shown to exert multiple beneficial effects in the prevention and treatment of diseases as well as neuroprotective effect in experimental models (Kim et al., 2015).

Accumulated evidence suggests that saponins have significant neuroprotective effects on the attenuation of central nervous system disorders such as stroke, AD, PD and Huntington's disease (Sun et al., 2015). The proposed mechanisms of its neuroprotective function include antioxidation, neurotransmitter modulation, anti-apoptosis, anti-inflammatory, attenuation of Ca2 + influx, modulating neurotrophic factors, inhibition of tau phosphorylation

and regeneration of neural networks acting on the cellular microenvironment (Sun et al., 2015).

Cheng et al, when evaluating the peripheral nerve regeneration at a 15-mm interval of the rat sciatic nerve, treated with different doses of AS-IV, observed that after 8 weeks of treatment with 50 mM dose, the animals had a rate of successful regeneration and a significantly higher number of myelinated axons, unlike the high--dose (200 mM) AS-IV treatment completely reversed this positive effect and inhibited the ability to promote nerve growth and regeneration (Cheng et al. 2006).

According to the aforementioned study, the authors believe that administering excessive amounts of AS-IV can provoke adverse responses to the recovery of degenerate neurons. However, when given in appropriate doses, as reported in its findings indicate that it may promote neurite outgrowth.

Increased neurite length may probably improve brain function and memory in the PD model studied, and although the subcellular mechanisms underlying these pharmacological effects are not deepened, they believe that this set of characteristics favored by AS-IV treatment is similar to of neurotrophins. Neurotrop h i c factors capable of promoting the survival, differentiation and maintenance of neurons in the development of adult vertebrate nervous systems. For example, glial cell line-derived neurotrophic factor and dopamine neurotrophic factor are one of the most potent neurotrophic factors for dopaminergic neurons (Lindholm et al., 2007). Thus, it is suggested that AS-IV may exhibit similar effect to neurotrophic factors and antioxidant effect relevant to the pathogenesis of PD and other neurodegenerative diseases (Chan et al., 2009).

Previous studies have demonstrated that AS-IV may have neuroprotective effects through its antioxidant properties and promote axonal regeneration in the peripheral nerve and the reconstruction of neuronal synapses (Cheng et al., 2006; Luo et al., 2004). AS--IV assisted in the reconstruction of synapses even in damaged neurons (axonal atrophy and synaptic loss caused by the amyloid pepti-

de (Tohda et al., 2006).

Other studies have observed the neuroprotective action of AS-IV in experimental models in vivo and in vitro of neurodegenerative diseases. The study conducted by Kim et al., 2015 evaluated the effect of AS-IV on learning and memory deficits induced by chronic cerebral hypoperfusion in rats, at dosages of 10 and 20 mg / kg, daily for 28 days from the 5th week after bilateral occlusion of the common carotid artery. It was found that AS-IV acted on the antioxidant defense system and significantly attenuated the level of MDA in the hippocampus when compared to the control group with induction of pathology. This study, performed immunohistochemical marking to GFAP, important because this protein acts as a marker of activation of astrocytes and microglia, acting on the defense system, however when evaluated in cases of hypoperfusion these cells indicate inflammatory processes, since glial activation excessive and persistent is a characteristic of neuroinflammation and is considered a mediator for secondary damage, neuronal death and disease progression (Minghetti et al., 2005).

A recent study has shown that AS-IV acts on the proliferation and differentiation of grafted, cultured neural stem cells (NSC) from the hippocampus of rat embryos. Cells were treated and transplanted into the hippocampus of AD rat mice. In vitro, it was shown that 10-5 AS-IV induced NSCs to differentiate into GFAP and β-tubulin III, a marker that is related to microtubule formation and neuron structure (Haiyan et al., 2016) . Therefore, in agreement our findings also allowed us to observe increase of cells expressing GFAP superior to the untreated groups and we performed analysis of a specific marker that regulates the synaptic transmission and plasticity, the GAP-43, indicating that the AS-IV acts in the maintenance of plasticity mechanisms.

Pretreatment of AS IV significantly inhibited isoflurane--induced neural apoptosis in the hippocampus, also significantly attenuated oxidative stress and release of proinflammatory cytokines (Sun et al., 2016). AS-IV

has been shown to act well along with other drugs as reported by Yang (Yang et al., 2012). The study investigated the effects of AS IV and tetramethylpyrazine on the cerebral ischemic / reperfusion injury model in rats. The authors concluded that AS IV and tetramethylpyrazine played a key role in synergistic protection against focal ischemic brain reperfusion injury. Thus, it is possible to infer that the treatment of AS-IV administered in association with other herbal medicines or the development of supplements with AS-IV and other drugs could act synergistically on the morphology of the hippocampal cells

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7. Conclusion

Sciatic nerve grafting with the addition of AS-IV in the mouse transected marrow promotes neuroprotection in the dorsal root ganglion neurons.

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