Sciatic nerve-conditioned medium with the addition of methylprednisolone promotes morphological plasticity in cultured spinal cord neurons

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Abstract

Introduction: Trauma is an important cause of morbidity and mortality worldwide, constituting a growing problem that is affecting young people who are in good health and are economically active. Spinal Cord Injury (SCI) is often a result of trauma, and it can be minimized by the actions of neuroprotective drugs. Evidence shows that methylprednisolone (MP) is a strong anti-inflammatory component, fighting lipid oxidation and thereby reducing the degeneration of the nervous
system. Drugs associated with sciatic nerve-conditioned medium (SNCM), which provides an environment that is rich in substances that influence the growth of injured nerve fibres in the peripheral nervous system (PNS), have been studied. **Objective**: Within this neuropharmaceutical perspective, this study analysed the cellular plasticity of spinal cord neurons in the presence of SNCM rats before the addition of MP. Method: Cell morphology was assessed over 72 hours, and immunocytochemistry analyses were conducted for β-tubuline III and NF-200 **Result**: Morphological analysis showed evident trophic development in the group that received the SNCM in the presence of MP (p≤0.0001), and immunoreactivity for β-tubulina III and NF-200 was more evident. **Conclusion**: This study of the plasticity of neurons of the spinal cord opened prospective new techniques for cell therapy in the presence of SNCM and MP, which promotes neuroprotective action.

**Introduction**

Traumatic Spinal Cord Injuries (TSCI) is a major source of axonal injury in the general population, affecting approximately 500,000 people worldwide per year (Singh *et al*., 2014; Furlan *et al*., 2013). As one of the most devastating injuries of organic systems, they frequently result in disabilities that require care from a multidisciplinary team for a long period of time, affecting the life of not only the injured person but of his/her family as well as society (Lim *et al*., 2007; Noonan *et al*., 2014; Dvorak *et al*., 2014).

In general, TSCI are related to automobile accidents and affect mainly young males. However, recent studies have shown an increase in the incidence in older adults as a result of domestic accidents and falls (Singh *et al*., 2014; Noonan *et al*., 2012; Selvarajah *et al*., 2014). In the United States, for example, costs associated with TSCI exceed the range of 10 billion dollars annually (Krueger *et al*., 2013). In Brazil, public spending on this population is approximately 9 billion reais/year, nearly one-third of all investment in public health in the country (Frison *et al*., 2013).

Neuronal damage caused by...
TSCI is grouped into two categories. Primary injuries are caused by the initial mechanism of injury of the spinal cord (SC). Secondary injuries occur after the initial damage to blood vessels in the SC region. These injuries cause a decrease in microvascular blood flow, leading to severe biochemical changes, as well as ischemia and hypoxia, which exacerbate the primary damage and can destroy neurons nearby that were not damaged during the traumatic impact (Sekhon et al., 2001; Cerqueira et al., 2013). It is important to note that most of the damage to the neurons of the SC after acute injury is the result of ‘secondary degeneration’ as a result of uncontrolled deleterious inflammation and not the initial traumatic damage (Chehrehhasa et al., 2014).

The past few years have been marked by a significant increase in research related to the recovery of post-traumatic nerve tissue. Therapeutic efforts, such as surgical stabilization and decompression, hemodynamic handling, and pharmacological interventions, aim to prevent further neurological deterioration and facilitate functional neurological recovery (Dvorak et al., 2015). Among these pharmacological strategies a primary intervention strategy includes the use of methylprednisolone (MP), a corticosteroid used to inhibit inflammatory reactions and prevent secondary injuries. Its results, however, are controversial, which implies a need for further study this drug (Kwon et al., 2004).

This study analysed the neuronal plasticity of the SC in the presence of sciatic nerve-conditioned medium (SNCM) rats before the addition of MP.

Materials and Methods

Animals

Male Wistar rats of an approximate age of 45 days and neonates aged 2 days were used under the approval of the Ethics Committee on Animal Experimentation from the State University of Rio Grande do Norte (UERN), Protocol number 007/13, in accordance with the ethical principles adopted by the Brazilian Society of Laboratory Animal Science and according to law number 11,794,
the Arouca law, of the Ministry of Science, Technology and Innovation. After the breast feeding period, rats were kept in a vivarium of UERN in separate cages with adequate housing conditions with free access to food and water until they were of appropriate weight and age.

**Conditioning DMEM for Sciatic Nerve Explants**

Under laminar flow, 60-mm (P60) culture plates with lids were prepared with 5 mL of Leibovitz-15 medium (L-15: Gibco, USA). Animals were anesthetized (Ketamine and Xylazine of Agener Union) and subjected to extraction of the sciatic nerve after the dorsal region was shaved and local asepsis was used with 2% chlorhexidine. Surgical approaches were made in the posterior region, and the sciatic nerve was removed and placed in P60 with L-15 medium under aseptic surgical techniques with the aid of micro devices (scissors, forceps and retractors). All of the excess tissues (muscle, fat, and blood vessels) attached to the nerves were removed under magnification by a SZ61 stereomicroscope (Olympus, Japan). Next, the epineurium of the nerves was removed under magnification via microsurgical techniques. The dissected nerves were segmented into explants that had a length of 2 mm length. Under laminar flow, the nerve fragments were placed in 60-mm plates with 1.5 mL of low Knockout Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA) plus 10% foetal bovine serum and 0.1% gentamicin, with a medium called D-10. Excess medium was removed from the explants so that they would not be floating or submerged. The D-10 medium from these cultures was changed two times per week, and the explants were transferred to a new plate with fresh medium 1 time per week. The medium was changed once and then disposed. This procedure allowed for an adequate nutrient supply to the explants and an analysis of their reactivity (Figure 1A and B). At this stage, SNCM (100 µl/plate) was collected for inoculation into the SC cell culture.
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**Figure 1**: Sciatic nerve explants showed initial migration cellular. The cells migrate from the explants, allowing cellular activity with substance production, which are secreted in the culture medium (constituting an extracellular matrix), thus enabling the collection of conditioned medium for the treatment of spinal cord neurons.

**Isolation and Spinal Cord Culture**

The animals were killed with an overdose of anaesthetic isoflurane inhalation (Isoforine®) (Cristália, Brasil). The animals were dissected under aseptic conditions for the removal of SC, then was held in a conical tube with L-15, followed by the SC removal. Under laminar flow, 60-mm culture plates were prepared with cell culture medium. The culture medium used was D-10. Unattached cells and residual non-adherent red blood cells were removed after 24 hours of washing with PBS. After 1 week in treatment the cells were resuspended from the plates with trypsin-EDTA and subjected to the 3 cycles of centrifugation with washes. Then, the cells of the spinal cord were plated in 24-well plates (1×106 cells/well) and were observed after 72 hours. With this procedure, it was possible to assess the adhesion and proliferation of SC cells in the following groups: group 1: SC cells + SNCM and group 2: SC cells + SNCM + MP (1μg/10μl).

The cell count was done using phase microscopy in 9 non overlapping fields at 10x magnification with a CKX41 microscope (Olympus, Japan). Photomicrographs of the 2 groups were performed at 72 hours. The cell morphology was observed using phase contrast microscopy at 10x magnification (Figure 2). After 72 hours of cell observation, we proceeded with immunocytochemistry for neuronal definition, the cellular morphometric (area and perimeter) was performed.

**Figure 2**: Spinal cord cells in culture after cell confluence.
Immunofluorescence Staining

By day three (72 hours), the cells had adhered to the plates, the medium was removed, and the cells were washed in two steps of five minutes each in PBS 0.1 M, pH 7.4; the cells were then fixed in paraformaldehyde (PFA) 4% for thirty minutes and washed again in three baths of PBS (five minutes each). Then, the cells were treated with 0.5% Triton (Sigma) for 10 minutes and washed in PBS. Subsequently, blocking of nonspecific sites was performed for 30 minutes in a PBS 0.1 M solution containing 0.2% Triton and 1% cattle serum albumin (CSA).

Plates were incubated for 2 hours at room temperature with anti-rabbit β-tubulin III (Millipore, 1:500) and anti-mouse NF-200 (Abcam, 1:1200). Upon completion of this step, cells were washed in PBS (0.1 M, pH 7.4) for five minutes and incubated for 1 hour with an anti-rabbit or anti-mouse secondary antibody produced in donkeys (Jackson, USA) conjugated to fluorophore AlexaFluor 594 or FITC and kept under refrigeration with the absence of light. After a secondary incubation, cells were washed with PBS for five minutes and immediately examined with a fluorescence microscope (Eclipse E200, Nikon) and then with a second fluorescence microscope (Eclipse Ni, Nikon). Photomicrographs were taken with Moticam 3.0 and 5.0 (Motic) digital cameras increased by 4x, 10x and 20x in 9 fields in a predetermined sequence on each plate. The presence of fluorescent staining was recorded in SC cells, taking care to examine the subcellular, cytoplasmic, and nuclear compartments.

Statistical Analyses

Two independent investigators calibrated (kappa=0.94) the counted cells per field in absolute numbers, using cell cultures of at least 3 different experiments with 10x magnification. The Motic Images Plus 2.0 (Motic) software was used for morphological observation (area in µm² and perimeter in µm), Image J software was used for cell counting, and Adobe Photoshop CS6.0 (Adobe) software was used to fix the minimum brightness and contrast of the photomicrographs. The database search was built on the SPSS pla-
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tform software (Statistical Package for Social Sciences) version 22.0, with a subsequent consistency check of typing. After the final structure of the database was completed, a descriptive analysis of all data was initially performed. The data were statistically compared by analysis of variance (ANOVA) with Fisher’s and Bonferroni tests and considered significant when $P < 0.05$.

**Results**

When comparing the mean area (Figure 3) and perimeter (Figure 4) of neuronal cells observed after 72 hours, the group 2 (SC+SNCM+MP) was superior to group 1 (SC+SNCM) ($p \leq 0.01$).

**Figure 3.** Average area of neuronal cells in the groups SNCM and SNCM plus FGF-2. Morphological analysis showing evident trophic development in the group that received SNCM in the presence of FGF-2, when compared to the group without FGF-2. Means $\pm$ S.E.M. $\ast p < 0.05$, $\ast\ast < 0.01$ and $\ast\ast\ast < 0.001$ according to ANOVA and post-test Fisher’s and Bonferroni.

**Figure 4.** Average perimeter of neuronal cells in the groups SNCM and SNCM plus FGF-2. Morphological analysis showing evident trophic development in the group that received SNCM in the presence of FGF-2, when compared to the group without FGF-2. Means $\pm$ S.E.M. $\ast p < 0.05$, $\ast\ast < 0.01$ and $\ast\ast\ast < 0.001$ according to ANOVA and post-test Fisher’s and Bonferroni.

When comparing the mean area (Figure 5) and perimeter (Figure 6) of glial cells observed after 72 hours, the group 2 (SC+SNCM+MP) was superior to group 1 (SC+SNCM) ($p \leq 0.01$).
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**Figure 5.** Average area of glial cells in the groups SNCM and SNCM plus FGF-2. Morphological analysis showing evident trophic development in the group that received SNCM in the presence of FGF-2, when compared to the group without FGF-2. Means ± S.E.M. ëp<0.05, ëë<0.01 and ëëë<0.001 according to ANOVA and pos-test Fisher’s and Bonferroni.

The group treated with SNCM in the presence of MP showed more evident immunoreactivity for β-tubulin (Fig. 7A) and NF-200 (Fig. 7B) compared to the other groups.

**Figure 6.** Average perimeter of glial cells in the groups SNCM and SNCM plus FGF-2. Morphological analysis showing evident trophic development in the group that received SNCM in the presence of FGF-2, when compared to the group without FGF-2. Means ± S.E.M. ëp<0.05, ëë<0.01 and ëëë<0.001 according to ANOVA and pos-test Fisher’s and Bonferroni.

**Figure 1:** Relationship between the PSA level and each age group.
**Discussion**

In this research, experimental environments created for nervous system fragments simulated an in vivo nerve injury. Thus, the explants became reactive and naturally secreted factors that made a favourable environment for post-traumatic nerve regeneration possible.

Morphological changes in the most obvious experimental groups are theoretically reflexes of a microenvironment that is conducive to cell growth. We note that the use of MP potentiated the neuroplastic effects of neurons and induced greater neuroprotective effects, enhancing cell viability, which was most significant 24, 48 and 72 hours after cultivation when compared to other groups. This finding showed that the substance had a direct effect on the growth of neurons in the earlier periods. Thus, we emphasize the direct use of MP in cases of trauma to the nervous system, thus minimizing the side effects of other routes of administration, which are discussed in the literature at a large scale.

Such an environment depends upon the secretion of neurotrophic factors, extracellular matrix components and cell adhesion molecules (Shields *et al*., 2000; Dezawa *et al*., 2001).

In this aspect, the groups treated with SNCM in the presence of methylprednisolone better highlighted neuronal growth with a rapid morphological plasticity for 72 hours. This growth was likely due to the nutritional contributions of SNCM, which creates a cellular environment that is conducive to cell regulation, promoting growth and enabling the support of neurite survival for nervous system regeneration (Hall, 2001), because Schwann cells retain their biochemical properties during regeneration in the CNS environment (Chen *et al*., 2005; Schwab, 2005; Guzen *et al*., 2009; Guzen *et al*., 2012). Corroborating Williams and Bunge (Williams *et al*., 2012), PNS cells, when implanted in the SCI, can support the regeneration of axons, assist in the myelination of damaged axons, and reduce secondary damage to tissues around the site of the initial injury, thus improving limb movement.
If the transplant is combined with additional treatments, such as MP, neurotrophins, among others, locomotion is improved. Once MP inhibits axonal damage and changes in the integrity of myelin, alterations are found in oedema secondary to SCI (Kozler et al., 2011; Bracken et al., 1984; Bracken, 2001), that are related to the reduction of autophagic expression of neurons after injury (Chen et al., 1976).

Combined treatment with Tanshinone IIA + MP reduce the expression of pro-apoptotic factors (caspase-3) and promote neuron survival in vivo and in vitro, thereby promoting neuroprotective effect by reducing apoptosis and inflammation caused by SC injury (Yao et al., 2017). Moreover, Cell death after SC primary injury was attenuated by pre-treatment with two known neuroprotective agents: the AMPA/KA blocker CNQX and MP (Krassioukov et al., 2002).

**Methylprednisolone** administration improves axonal regeneration into Schwann cell grafts in transected adult rat thoracic SC, being thus MP improves axonal regeneration from both SC neurons into thoracic SC grafts, possibly by reducing secondary host tissue loss adjacent to the graft (Chen et al. 2009).

When MP is administered following SC contusion in the rat, lesion volumes at 24 h are reduced (Constantini et al., 1994) and the percentage of spared tissue at 29 days is increased (Behrmann et al., 1994). In a case study (Bunge, 1994) of a contused cord from a person who had received the early high-dose MP treatment, the restricted linear zone of injury (no more than 2 cm) was striking and remarkable compared with cords from untreated injured persons.

The major neuroprotective effect of MP is believed to be related to its ability to inhibit oxygen free radical induced lipid peroxidation (Hall et al., 1992), peroxidation is an early event in the secondary destruction of injured SC tissue (Anderson & Hall, 1994). MP has other demonstrated mechanisms of neuroprotection as well, including inhibition of lipid hydrolysis (arachidonic acid release) and eicosanoid formation, maintenance of blood flow
and aerobic energy metabolism, reversal of intracellular Ca2+ accumulation, reduction in neurofilament degradation, enhancement of neuronal excitability and synaptic transmission, and potent anti-inflammatory activity (Hall et al., 1992; Anderson & Hall, 1994; Perot, 1994; Young et al., 1992; Young et al., 1994).

Thus, it is understood that MP provides support for neuronal cells and that it is important in the repair process to reduce the deleterious effects of nerve damage and to improve functional performance, a fact that is corroborated in the work of Zivadinov et al. (2008), which showed that the application of MP reduces sclerotic lesions, suggesting that chronic applications of MP lead to significant reductions in nervous lesions.

**Conflict of Interest Statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Conclusion**

We demonstrate progress in understanding the components of the nervous-stimulating cellular environment. We enabled neuroprotective activity in groups over a short period of time, thus ensuring their differentiation potential under in vitro exposure to appropriate stimuli and providing a higher survival of the cells. These findings reiterate that MP and conditioned medium should be used in research. The results emphasize that the manipulation of the microenvironment at the wound might amplify the regenerative capacity.

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References


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