Sciatic nerve-conditioned medium promotes trophic induction in spinal cord cultured cells

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Abstract

The fibers in the peripheral nervous system are frequent targets of injuries, being distributed throughout the length of the body. Such lesions culminate in motor, sensory and autonomic changes, thus recent evidence shows the influence ideal environment for growth of nerve fibers damaged in the Central Nervous System (CNS). Our objective was to verify this cell behavior in the central sphere, analyzing cellular plasticity of the spinal cord (SC) in the presence of sciatic nerve-conditioned medium (SNCM) from neonatal rats. 12 animals were used, 06 of those aged 45 days, and the other 06 aged 02 days, and it was

Keywords:

Spinal Cord
Conditioned medium
Sciatic nerve
Plasticity
approved by the Ethics Committee on Animal Experimentation nº007/13. It was inoculated SNCM from explants of the sciatic nerve of the animals in the SC neurons culture aiming the trophic behavior of these cells by morphometric analysis and immunocytochemistry for microtubule-associated protein-2 at the end of 72 hours. The neurons and glial cells of the SC showed a typical morphology and rapid expansion, particularly in samples that higher dosage of SNCM were exposed when compared to the group devoid of SNCM (p≤0,0001). However, this study improves the understanding of the plasticity of neurons and glial cells in the SC and facilitates understanding in the search for better techniques with vehicle management and cell therapy in the use of disease and trauma in the CNS. The trophic role of the SNCM made it possible to register the patent in the organ of the National Institute of Intellectual Property (nº BR 1020160162289).

**Introduction**

Central Nervous System (CNS) disorders are complex, often resulting in permanent loss of motor functions, sensitivity and / or cognitive functions such examples in Multiple Sclerosis, Parkinson’s, Alzheimer’s, stroke and traumatic injuries, and also bring severe social and emotional consequences (Ma et al, 2014; Almeida et al, 2016; Leal-Filho et al, 2008). Some acute neurodegenerative diseases, such as stroke and traumatic lesions of the brain and spinal cord have a high global incidence and high rates of morbidity and mortality. The stroke sequelae lead the chronic disabilities causes of numbers (approximately 795,000 annually) in the United States. Approximately half of the patients who survive are left with the reduced mobility and unable to walk unassisted (Ma et al, 2014; Go et al 2014). In addition estimates of the World Health Organization (WHO) shows that in 2040 neurodegenerative diseases will exceed the cancer as the leading cause of death in industrialized countries.

Multiple pathophysiological mechanisms can contribute to CNS injury, including death by
Sciatic nerve-conditioned medium promotes trophic induction in spinal cord cultured cells

apoptosis and neuronal necrosis, scarring process by reactive astroglialosis, production of inhibitory factors by extracellular matrix molecules, ischemia, excitotoxicity, oxidative stress, axonal damage and inflammatory or demyelinating processes (Fitch & Silver, 2008). The poor tissue regeneration is assigned to a total absence of growth factor in CNS and a suitable extracellular matrix (ECM) substrates which would allow this growth6 and also the presence of inhibitory molecules of axonal growth at the site of the lesion and its proximity (Fitch & Silver, 2008; Machado & Haertel, 2014; Tuttolomondo et al, 2014). Glial scars formed by astrocytes and microglia, physically block tissue regeneration, and also can release inhibitors growth factors, such as inhibitor of neurite outgrowth (Nogo) (Chen et al, 2002).

The Peripheral Nervous System (PNS) is mainly formed by nerves, which are bundles of axons, enveloped by Schwann cells (SCs), maintained by the endoneurium and amorphous extracellular matrix (ECM), capillaries, fibroblasts, and mast cells, as well as the perineurium and epineurium (Lundy-Ekman, 2008; Pellitteri et al, 2006). The SCs play an important role as they are the source of different trophic substances and have a great neurotrophic activity (Pellitteri et al, 2006). Several neurotrophic factors are released and act together after a peripheral nerve injury in order to stimulate neural regeneration; include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5, ciliary neurotrophic factor, insulin growth factors I and II (IGF-I and IGF-II), fibroblast growth factor (FGF-2), neuregulin 2, among others (Pellitteri et al, 2006; Du et al, 2010; Lingxiao et al; 2015; Kim et al, 2013; Nakano et al, 2016). The functional regenerating axons most frequently occurs in the PNS, in part because the production of nerves growth factor by CSs contribute to the recovery of peripheral axons (Machado & Haertel, 2014; Lundy-Ekman, 2008; Kim et al, 2013).

This study aimed to analyze the cellular plasticity of Spinal Cord (SC) in the presence of different dosages of sciatic nerve con-
conditioned medium (SNCM) from neonatal rats. The conditioned medium created from Sciatic Nerve (SN) fragments and containing ECM components and neurotrophic factors, was inoculated in SC neurons and glia culture observing the trophic behavior of these cells.

**Materials and Methods**

**Animals**

Male Wistar rats at an approximate age of 45 days and neonates aged 2 days were used in this study under the approval of the Ethics Committee on Animal Experimentation from the State University of Rio Grande do Norte (UERN), Protocol number 007/13. All experiments were conducted in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No.8023, revised in 1978).

**Sciatic nerve-conditioned medium (SNCM)**

Animals were anesthetized with ketamine and xylazine mixture (Agener União, Brasil) and subjected to extraction of the sciatic nerve, which was then transferred to 60mm plates with Leibovitz L-15 medium (L-15: Gibco, USA). The other tissues (muscle, fat, and blood vessels) attached to the nerves were removed under magnification using a SZ61 stereomicroscope (Olympus, Japan). Further, the epineurium of the nerve was removed and fragments of 2 mm in length were obtained, where they were placed in 60mm plates with 1.5 mL of knockout Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA) plus 10% foetal bovine serum and 0.1% gentamicin (Culture medium D-10). The medium was changed two times a week and the explants were transferred to a new plate with fresh medium once a week. This procedure allowed for reasonable nutrient supply and adequate analysis of their reactivity (Figure 1). At this stage, SNCM (various volumes) was collected for inoculation into the spinal cord cell culture.
Sciatic nerve-conditioned medium promotes trophic induction in spinal cord cultured cells

**Isolation and Spinal Cord Culture**

The animals were sacrificed with a lethal dose of anaesthetic (isoflurane inhalation - Isoforine® - Cristália, Brasil). The animals were dissected under aseptic conditions for the removal of spinal cord, followed by its immersion in L-15 medium. Spinal cord cells were placed (1 × 106 cells/plate) in 24-well plates in the presence of 1.5 ml of D-10 and were observed over three time periods: 24, 48, and 72 hours. With this procedure, it was possible to assess the adhesion and proliferation of spinal cord cells in the following groups: 1 - spinal cord cells + D-10, 2 - spinal cord cells + 50μl of SNCM, 3 - spinal cord cells + 100μl of SNCM and 4 - spinal cord cells + 200μl of SNCM. In this way, group 1 remained with 1.5 ml of D-10, group 2 was removed 50 μl of D-10 and added 50 μl of SNCM, proceeding in the same way for the other volumes of group 3 and 4.

The cell count was performed by phase microscopy in 9 non overlapping fields at 10x magnification using a CKX41 microscope (Olympus, Japan). The cell morphology was observed using phase contrast microscopy at 10x magnification. After 72 hours, the immunocytochemistry was performed for microtubule-associated protein-2 (MAP-2).

**Immunofluorescence Staining**

By day three (72 hours), the cells had adhered to the plates and the medium could be easily removed. Cells were washed following two steps of five minutes each using PBS 0.1 M, pH 7.4. Cells were then fixed in paraformaldehyde (PFA) 4% for thirty minutes and washed again in three baths of PBS (five minutes each). Further, the cells were treated with 0.5% Triton (Sigma) for

**Figure 1.** Sciatic nerve explants (arrow) showed initial migration of fibroblasts and Schwann cells (arrowhead).
10 minutes and washed with PBS. 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-mouse MAP-2 (Sigma, 1:2000). 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 anti-mouse secondary antibody produced in donkeys (Jackson, USA) conjugated to AlexaFluor 594 and kept under refrigeration in 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).

Statistical Analyses

Two independent investigators calibrated (kappa=0.94) the counted cells per field in absolute numbers, using cell cultures of at least 8 different experiments with 40x magnification. The Motic Images Plus 2.0 (Motic) software was used for morphometric analysis (area in \( \mu m^2 \) and perimeter in \( \mu m \)) and Adobe Photoshop CS6.0 (Adobe) software was used to fix the minimum brightness and contrast of the photomicrographs. Statistical analyses were performed using the analysis of variance (ANOVA) and significant interactions were followed-up with a Tukey and Bonferroni post-test comparison. All statistical analyses were performed using SPSS 22, and significance was set at \( P < 0.05 \).

Results

With the monitoring of the cells for 72 hours, it was verified that the populations suffered a process of trophism progressively from the first day until the last day of observation in all the experimental groups.

After measuring the area of the glial cells, it was observed that over the three days of observation of groups 1, 2, 3 and 4 the glial cells increased their area, an effect more evident in the groups treated with SNCM. On days 1, 2 and 3 of observations (24, 48 and 72 hours), the mean area of the cells of groups 3 and 4 were higher than that of group 1 (***\( p = 0.000 \)). No differences were observed between group 2 in
Sciatic nerve-conditioned medium promotes trophic induction in spinal cord cultured cells

In the analysis of the measurement of the perimeter of the glial cells, it was observed that over the three days of observation of groups 1, 2, 3 and 4 the glial cells increased their perimeter, an effect more evident in the groups treated with SNCM. On observation days 1, 2 and 3 (24, 48 and 72 hours), the mean perimeter of the cells of groups 2, 3 and 4 were higher than those of group 1 (**p = 0.000). Regarding the groups treated with SNCM, it was observed that group 4 had a larger cell area when compared to groups 2 and 3 in the analysis periods. Similar behavior was also observed when we purchased group 3 in relation to group 2 (Figure 2B).

After measuring the area of the neuronal cells, it was observed that over the three days of

Figure 2. Morphometric analysis of the glial cell area (2A) and perimeter (2B) showed that the SNCM treatment promoted a plastic effect in the glial cells, being more evident in the group treated with 200μl SNCM when compared to the group without SNCM and to the other groups treated with the volumes of 100 and 50μl of SNCM, according to ANOVA - Tukey and Bonferroni.

Figure 2.

Morphometric analysis of the glial cell area (2A) and perimeter (2B) showed that the SNCM treatment promoted a plastic effect in the glial cells, being more evident in the group treated with 200μl SNCM when compared to the group without SNCM and to the other groups treated with the volumes of 100 and 50μl of SNCM, according to ANOVA - Tukey and Bonferroni.
observation of groups 1, 2, 3 and 4 neuronal cells increased their area, an effect more evident in the groups treated with SNCM. On days 1, 2 and 3 of observations (24, 48 and 72 hours), the mean area of the cells of groups 2, 3 and 4 were higher than that of group 1 (**p = 0.000). Regarding the groups treated with SNCM, it was observed that group 4 had a larger cell area when compared to group 2 in all periods of analysis and group 3 in 48hs and 72hs. When comparing group 3 with group 2 we observed that group 3 had a larger cell area within 48 hours of analysis (**p = 0.005) (Figure 3A).

In the analysis of the measurement of the perimeter of neuronal cells, it was observed that during the three days of observation of groups 1, 2, 3 and 4 neuronal cells increased their perimeter, an effect more evident in the groups treated with SNCM. On observation days 1, 2 and 3 (24, 48 and 72 hours), the mean perimeter of the cells
of groups 2, 3 and 4 were higher than those of group 1 in 24 hours (**p = 0.004) in 48 and 72 hours (**p = 0.000). No differences were observed between the groups treated in the 24-hour period. However, group 4 had a larger cell area when compared to groups 2 and 3 in 48hs and 72hs (**p = 0.000). Similar behavior was observed when comparing group 3 in relation to group 2 (Figure 3B).

After 72 hours of observation, the immunocytochemistry of the 4 experimental groups was performed. The fluorescence microscope filters were closed to validate the labeling. The populations of the cells of the 4 groups expressed MAP-2. However, the expression of MAP-2 resulted in different intensities, being more evident and with more plastic cells in the groups treated with SNCM (arrow), an effect that was potentiated by 200μl of SNCM (Figure 4).

**Figure 4.** Photomicrographs of cultured spinal cord cultured cells treated with D-10 (A) medium, SNCM 50μl (B), SNCM 100μl (C) and SNCM 200μl (D), immunoreactive to MAP-2. Illustration shows more trophic cells (arrows), with more marked intensity in the group that received addition of 200 μl of SNCM when compared to the group lacking SNCM and to the other treated groups.

**Discussion**

These results may provide a cellular explanation for the enhanced plasticity seen in rodent spinal cord culture models administered SNCM.

In our study, the medium created from nerve fragments simulated a nerve injury situation. Thus, the explants became reac-
tive and naturally secreted factors that would allow a favorable environment for nerve regeneration after trauma. The histology organization of the SNCM possibly explains the high reactivity and rapid deposition of a possible neurotrophic factor in the conditioned medium. The peripheral lesion microenvironment simulated by SNCM explants can justify the speed of neuronal and glial plasticity, and this effect is enhanced by the increased volume of SNCM.

In order for the cells to function properly, they must be organized in space and mechanically interact with the environment around them. They must have a correct conformation, being physically robust and appropriately structured internally (Alberts et al., 2010). In addition, every cell must be able to reorganize its internal components as a result of the processes of growth, division and / or adaptation to changes in the environment (Alberts et al., 2010). Thus, the most important aspect for cell survival in a receptor tissue is the microenvironment around it. Initially, this aspect is related to the cell surface expression of adhesion markers which interact with extracellular matrix components. Added to the paracrine effects of growth factors secreted by surrounding cells, the microenvironment allows conditions for the survival, migration, invasion and tissue differentiation (Caddick et al., 2006).

Cultures of cells when grown in the presence of SNCM showed more noticeable changes compared to cultures devoid of such treatment, allowing a more obvious identification of the morphology of glial and neuronal cells, thus resulting in plastic effect. An important aspect in our study was the area and cell perimeter because these were parameters primarily selected for morphological description of the cells throughout the days, and presented significant relevance in the statistical analysis over the three days of observation. Control of cell size is an important aspect of cellular growth and control of morphology is essential for the differentiation and development of organs and tissues. Neurons of the SC showed a typical morphology
and rapid expansion, particularly in the samples that were exposed to SNCM. These cells cultured in the presence SNCM also showed a higher expression of MAP-2 protein, which relate to the stability of microtubules of neuronal and glial cells, preventing their cleavage (Caddick et al, 2006). Cells that make overexpression of MAP-2 form bundles of stable microtubules with an ample spacing (Alberts et al, 2010). The inhibition of MAP-2 in hippocampal and cerebellar neurons prevents the expression of neurite growth, and the inhibition of this protein after the formation of neurites results in disorganized microtubules and reduces the number of neuritic processes (Monteiro et al, 2011).

In our study, MAP-2 expression resulted in different intensities and were more evident in treated groups, in other words, increasing marking with increasing dosage of SNCM and therefore, the group 4, which was added a higher concentration of SNCM (200μL), we found a greater neuronal and glial growth (cells area and perimeter).

Villegas et al, (1995, 1996) showed that the SNCM shows high neurotrophic activity capable of producing rapid neuronal differentiation of PC12 cells and increase cell survival and differentiation of dorsal root ganglion and ciliary ganglia of chicken embryos. They also showed that the cultured SCs from sciatic nerve are the main source of neuroregulin, protein identified as a major neurotrophic factor in the conditioned medium related to differentiation of PC12 cells, that associated with fibroblasts and perineural cells and some extracellular matrix components such as collagen, fibronectin and proteoglycan. Moreover, treatment with conditioned medium of dental pump cells significantly induced neurite outgrowth from PC12 cells (Kudo et al 2015), neural stem cell-conditioned media promote neuroprotective effect in vitro model of Huntington’s disease (Lim et al, 2008) and conditioned medium of the primary culture of choroid plexus epithelial cells enhances neurite outgrowth and survival of hippocampal neurons (Watanabe et al, 2005).

In SNCM we can still find a variety of known and unknown molecules that can promote or
inhibit neuronal polarity and facilitate or block the tissue regeneration process (Longart et al., 2009). Some molecules present in SNCM such as neuregulin and endogenous precursor of NGF (pro-NGF) have the ability to promote neuronal differentiation of PC12 cells by the growth of dendrites (Longart et al., 2009; Villegas et al. 2000), and induce functional changes enabling and modulating voltage-gated calcium, sodium and potassium channels and so have a pro-neuritogenesis effect and increase neuronal cell survival (Longart et al., 2009; Villegas et al. 2000; Castillo et al., 2006).

Du et al., (2010) showed that the SNCM has a higher level of expression and concentration of NGF and also a higher neurotrophic activity compared with conditioned medium of the optic nerve. And these results suggest that the PNS and the CNS have different levels of neurotrophins expression which could partly explain the lack of CNS regeneration.

**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**

This study and its results allow us to suggest that the sciatic nerve fragments in culture medium secrete molecules which have a trophic and regenerative effect on glia and CNS neurons in vitro. Many questions remain to be answered with regard to understanding the mechanisms of action of neurotrophic factors presented in the conditioned medium as well as the role of yet unknown molecules secreted by Schwann cells. In this way it was hoped that the explants became reactive and naturally produced and released factors that would enable a conductive environment to post-traumatic regeneration.
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