Caroline Nesello

EFEITOS DA NICOTINA NA LESÃO MEDULAR TRAUMÁTICA: UMA ANÁLISE *EX VIVO*

Dissertação apresentada à Universidade de Caxias do Sul, para obtenção do Título de Mestre em Ciências da Saúde.

Caxias do Sul

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Caroline Nesello

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"Comece fazendo o que é necessário, depois o que é possível, em breve estarás fazendo o impossível."

São Franscico de Assis

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Esta dissertação de Mestrado Acadêmico Stricto Sensu é apresentada no formato exigido pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul. A mesma é constituída da secção de “Introdução com referências bibliográficas”, a inclusão do artigo original submetido/publicado em periódico Qualis A na classificação da Coordenação de Aperfeiçoamento de Pessoal em Nível Superior (CAPES), e as “Considerações Finais e Perspectivas”.

1 INTRODUÇÃO

O trauma raquimedular (TRM) é uma patologia grave resultante de lesões na medula espinhal que provocam alterações temporárias ou permanentes na sensibilidade, função motora e autonômica. E que comumente acarretam sequelas irreversíveis e progressivas, repercutindo física, psíquica e socialmente na vida do paciente. Define-se por ser o resultado de uma força física que supera a capacidade de proteção e sustentação da coluna vertebral, ocasionando uma compressão e até mesmo a laceração da medula espinhal[1].

O TRM provoca diversas modificações celulares e moleculares, esses eventos têm sido subdivididos em duas etapas, a lesão primária, ocasionada por destruição celular e danos nos axônios por meio de fatores mecânicos, e a lesão secundária decorrente das respostas bioquímicas que aumentam a extensão do dano tecidual[2].

Esta patologia possui incidência mundial anual estimada de 40 a 80 casos por milhão de habitantes[3], são escassos os estudos que avaliaram a epidemiologia do TRM no Brasil, estima-se a ocorrência 40 novos casos por milhão de habitantes[4]. Definitivamente, trata-se de uma patologia que possui significante impacto socioeconômico e que por fim acaba sobrecarregando o sistema de saúde em virtude das frequentes internações e complicações que podem se manifestar concomitantemente[5].

Atualmente o número de pessoas que buscam por uma vida saudável vem aumentando gradativamente, e o tema “Tabagismo” tem chamado atenção por conta dos seus efeitos deletérios. Neoplasias, doenças cardiovasculares e respiratórias associadas ao consumo de cigarro são bem documentadas; entretanto não se têm informações referentes às complicações cirúrgicas que ele acarreta, bem como dos seus efeitos associados ao TRM[5-6].

A nicotina é a principal substância causadora de dependência do cigarro[6-7], e também o componente vasoativo mais relevante da fumaça do tabaco, sendo uma substância alcaloide, inodora e incolor que, quando inalada ou injetada, pode acarretar o aumento da produção de catecolaminas e resultar em vasoconstrição e diminuição da perfusão tecidual. A presença da nicotina na circulação sanguínea é correlacionada com um nível aumentado de necrose tecidual prejudicando o processo de cicatrização, contudo, escassos estudos reportam os efeitos da nicotina

diretamente na lesão medular.

O uso de ratos como modelo experimental de trauma raquimedular possui protolocos bem estruturados e difundidos em diversos grupos de pesquisa, no entanto requerem cuidados substanciais no período pós operatório, investimento elevado e normalmente mais demorados, sendo assim, métodos alternativos vem se tornado mais atrativos. Além de respeitar o princípio dos 3R’s (*replacement, reduction and refinement*) o modelo experimental *ex vivo* de TRM permite estudar as alterações fisiopatológicas mimetizando as condições *in vivo*, preservando a conectividade citoarquitetural e neural, proporcionando avaliação celular em tempo real.

O presente estudo propõe verificar o efeito da nicotina na lesão medular pós-traumática utilizando o modelo de cultivo *ex vivo* de TRM, o qual utiliza fragmentos da medula espinhal de ratos em cultivo celular e posterior compressão por impacto para mimetizar o trauma. Este modelo nos permite analisar eventos bioquímicos e celulares para elucidar as interfaces que caracterizam o microambiente alvo de estudo.

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3 ARTIGO

Acute nicotine exposure evokes spinal cord cell death in an *ex-vivo* traumatic spinal cord injury model

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Abstract

Nicotine is a psychoactive drug that has deleterious effects. However, there is little literature concerning its effects on spinal tissue. Spinal cord injury (SCI) resulting from spinal lesions that cause changes in sensitivity, motor and autonomic neuronal function, which can lead to irreversible life-changing neurological damage. Nicotine has been linked to increased tissue necrosis, resulting from vasoconstriction and decreased local perfusion. In order to understand this relation, this work investigated the effect of nicotine on spinal tissue after SCI, using the *ex-vivo* model of spinal tissue from *Wistar* rats. The tissues were incubated for 3 days, followed by the protocols of nicotine induction (10 mM for 3 days acute exposition), and SCI compression by impact from a height of 25 mm and weight of 0.5 grams in the center of the medullary tissue section and then 14 days of follow-up. Samples of the supernatant were collected at 24h up to 14 days for LDH analysis. By the end of the experiment, MTT tests, nuclear morphology by DAPI and LiveDead cell assays were performed. The tissues that were exposed to nicotine and then suffered SCI had less cell viability and showed higher levels of LDH, when compared to the other groups. Since SCI is a limiting condition and nicotine is a potentially vasoactive substance, it was possible to assess its effects on medullary tissue and verify that both hampered tissue recovery. This study has important implications and encourages the elucidation of the nicotine mechanism in SCI.

Keywords: Nicotine; spinal cord injury; cell death; *ex-vivo* model.

1. Introduction

Spinal cord injury (SCI) is a serious pathology resulting from spinal cord damage that provokes temporary or permanent changes on motor sensibility and autonomic functions. It usually entails irreversible and progressive sequelae with physical, psychological and social impacts on the life of patients. It is defined as the result of a physical force that surpasses the capacity of protection and support to the spine, causing compression, or even laceration of the spinal cord (McDonald and Sadowsky). SCI leads to several cell and molecular alterations, and these events have been subdivided into two stages, the primary lesion, caused by cell destruction and damage in the axons by means of mechanical factors, and the secondary lesion resulting from biochemical responses which increase the range of tissue damage (Hillen et al.).

This pathology has a worldwide and annual estimated incidence of 40 to 80 cases per million inhabitants(2013). Due to frequent complications and hospitalization, they burden the health system and impact the social and economic sectors of the country.

Currently the number of people who seek a healthy life is gradually increasing, and the topic of "*smoking*" has been emphasized on account of detrimental implications. Many diseases such as neoplasias, cardiovascular and respiratory diseases, associated with cigarette smoking, are well documented; however, there is no information regarding the surgical complications that result from it, as well as its impact related to SCI (Kaur et al., 2018; Sachar et al.).

Nicotine is an alkaloid and vasoactive compound; and the main cause of addiction to smoking dependency (Badanavalu and Srivatsan, 2019; Bhatti et al.). When this alkloid is inhaled or injected it can produce catecholamines leading to vasoconstriction and reduction perfusion. Once nicotine is in the bloodstream it is correlated with tissue necrosis and delay of the healing process. However, there are few studies reporting the effects of nicotine on SCI.

Therefore, the current study proposes to look at the effect of nicotine on the SCI using

the TRM *ex-vivo* culture model.

2. Materials and Methods

2.1 Study Design

Experimental Study

This study looks at the effects of nicotine on SCI, through the *ex-vivo* modeling of spinal cord tissue, using the MASCIS Impactor device to create the SCI. The load device weighing 5 g was dropped from a height of 25mm, which corresponds to moderate SCI (Falavigna et al., 2018).

2.2 Animals

Male Wistar rats (N=6), 2-3 months old, weighting 120-160 g, were acquired from the Biological Models and Experimental Center of (CeMBE) Pontifical Catholic University of Rio Grande do Sul (PUCRS). During the set of experiments, the animals were kept in cages in the Laboratory of Animal Physiology and Experimentation at the University of Caxias do Sul (UCS), with 12- hour dark/light cycles. Humidity and temperature were maintained between 24 ± 2°C and 55 ± 15%, respectively, in a controlled environment. The rat received rodent pellet food and filtered water *ad libitum*, and they were also acclimatized to the installation for 7 day before the experiment. All the procedures and technical handling were done according to the Brazilian Guideline for Care and Use of Animals for Scientific and Educational Purposes (MCTI, 2013), following the advice on the welfare of laboratory's animals and ethical standards for experimentation presented in the Guide regarding the use and care of laboratory animals of the National Institute of Health (NIH) and the respective directives of the Animal Ethics Committee of the institution (CEUA-UCS), that approved the implementation of this project under protocol number 014/2018.

2.3 Spinal cord slice culture / *Ex-Vivo* Model

The *ex-vivo* tissue culture was prepared with spinal cord slices of the rats. These animals were euthanized with an anesthetic overdose of inhaled isoflurane and the skin of the dorsal region was shaved. Under aseptic conditions, the skin was incised on the musculature close to the midline of the dissected vertebral column and the spine was exposed. After the laminectomy was performed, the spinal cord was removed, and immersed into *Hanks' Balanced Salt Solution* (Gibco) in a sterile Petri dish kept on ice for cellular viability and tissue structure. Under laminar flow, cross-sectional slices, 350 µm thick were made, and the intact morphological spinal slices were transferred onto the Millicell culture plate inserts (0.4 µm, Millipore) using a sterile clamp on a six-well plate and immersed in 2 ml culture medium MEM with L-glutamine, 25% heat-inativated horse serum (HBS) and 1% of penicillin/streptomycin (Pandamooz et al., 2017). The culture plates were incubated in a 5% of CO_2 humidified incubator at 37°C. After 4 days in culture, the slices were assigned to four groups: (i) Control, (ii) Nicotine, (iii) SCI and (iv) Nicotine-SCI, with N=5 per group, in triplicate (Figure 1).

2.4 Nicotine Induction Protocol

Induction was performed by dilution of MEM plus Nicotine (Sigma) at a 10 mM concentration (Mahapatra S.K., 2009) in Nicotine only and nicotine-SCI groups beginning on the 5th day of culture. The acute exposure was maintained for 72 hours (until the 8th day) and the culture medium was changed daily.

2.5 SCI Protocol

The spinal cord injury was induced on the 9th day of culture, with a weight drop device, the MASCIS Impactor (Multicenter Animal Spinal Cord Injury Study), since it has a pendulum that when driven drops on the tissue and causes an injury. It was chosen to use the 5g pendulum, with a drop height of 25mm causing a moderate spinal cord injury (Falavigna et al., 2018). The three spinal cord slices of the only SCI and the Nicotine-SCI

groups were removed from the cell culture plate and put on a Petri sterile dish, positioned on the platform of the equipment and aligned with the pendulum. Each spinal cord slice was injured with the same weight and height. Subsequently, the slices were replaced in their respective wells and kept in the culture. The medium of culture slices was changed every two days (Figure 1).

2.6 Lactate Dehydrogenase Activity

The lactate dehydrogenase (LDH) activity assay (LDH Liquiform kit, Labtest, ref.86) was used to assess and quantify the cell injury, in order to observe the conversion of pyruvate into lactase to determine the quantity of LDH (LDH Liquiform kit, Labtest, ref.86) using the automatic analyzer (Mindray, BS-120). Samples were collected on days 1, 3, 5, 8, 10 and 14 of the medium tissue cultures of all groups. Later, the samples were centrifuged at 2300 rpm for 15 min and the supernatant was collected for analysis.

2.7 Cell viability tissue assay

The colorimetric assay with MTT was used to corroborate the cell viability of the spinal slices. The MTT salt of tetrazoliumwas used with a 10% v/v in a solution of 5 mg/ml (PBS) and exposed to the tissue. Next, the spinal cord slices were incubated during 3 hours in a humidified incubator at 37°C and 5% of CO_2. The metabolically viable cells react with MTT, a translucent yellow dye from formazan crystals, through the active mitochondria and other cell enzymes. Since it was dark blue giving a purple hue to the cytoplasm of the living cells that may be visualized under an optical microscope (Carl Zeiss) it was quantified by absorbance using equation: $Cell\ viability\ (\%) = (Abs_s/Abs_{control})100$; where Abs_s is the absorbance of tissue cells treated with nicotine and/or TRM injury and $Abs_{control}$ is the absorbance of tissue control cells (incubated with MEM only). The experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation. $P < 0.05$ was indicative of statistical significance versus control group.

2.8 Nuclear Morphology and Morphometric Analysis by DAPI

The 40,6-diamino-2-phenylindole (DAPI) staining was carried out to establish the nuclear morphology of the tissue cells. Briefly, on the 14th day the tissue cells were washed three times in PBS, and fixed with 4% formaldehyde at room temperature, for 48 h. The fixed tissues were then washed with PBS, permeabilized with 0.1% Triton X-100 in PBS and stained with a 600 nM DAPI solution (Santa Cruz, CA) at room temperature, for 24 h. The acquisition of pictures and analyses of the nuclear morphology of the tissue cells was done under a fluorescent microscope AXIOVERT II (Carl Zeiss MicroImaging GmbH, Germany) and its respective Zen Blue software. DAPI staining clearly delineates de nuclear morphology that allows the quantification of the Raw Intensity Density. Data from control tissue cells (untreated) are used to set the parameters of the normal population. The morphometric parameters were calculated considering 100 events in four regions of gray matter for each group, three times in triplicate. The experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation.

2.9 Live/Dead Cells Trial

The LIVE/DEAD Cell Viability Assay kit (Abbikine) was employed to identify and quantify dead cells in the tissue culture slices. Cell culture slices at 14 days of follow-up were incubated for cell labelling, following the manufacturer’s specification. In this assay, we load cells with Calcein-AM plus propidium iodide (PI), which penetrate the live (green fluorophore) and dead (red fluorophore, respectively, and count red/green fluorescente cells). Then, the slices were placed back into the incubator for 30 min to allow permeation of Calcein-AM and PI. Acquisition images were taken immediatly in the four regions of gray matter for each group, three times in triplicate. The image and data analyses were performed using an

inverted microscope setup for fluorescence microscopy AXIOVERTII (Carl Zeiss MicroImaging GmbH, Germany) and its respective Zen Blue software.

3. Results

3.1 *Spinal cord slices in ex-vivo culture: nicotine exposition and SCI*

Our set of experiments used fragments of the spine of adult rats in cell culture and subsequently compression by weight drop impact to simulate the SCI trauma. It has been clearly proved that adult tissue is more subject to cell damage in slice acquisition and culturing than embryonic tissue. This model enables us to investigate biochemical and cellular events to analyze the interfaces that characterize the microenvironment of the SCI. Besides respecting the 3 Rs tenet (*replacement, reduction and refinement*), the SCI ex-vivo experimental model allowed studying the pathophysiological changes simulating the *in vivo* conditions, preserving the neural and cytoarchitectural connectivity providing cell assessment in real time (Figure 2A). Based on these, the adult cultured spinal cord slices are a feasible model, since SCI occurs predominantly in adults and is characterized by a traumatic insult and secondary tissue damage. Highlighting the possibility of isolating viable resident spinal cord cells at the end of the experimental endpoint in all experimental groups proves the quality and survival of the *ex-vivo* model throughout this time (Figure 2B).

LDH activity was measured from the culture supernatant, which allowed the material to be collected at different times. The presence of a high LDH concentration in the culture meant greater tissue damage. Initially, the LDH concentration was high, as a result of tissue manipulation due to the transection and removal of the spinal cord. In a few days time, there was a reduction in LDH concentration, as the tissue slices recovered and the culture environment was established. In our model system, there were no statistical differences between Control, Nicotine and SCI groups in LDH activity. However, there was a significantly increased level in the group exposed to nicotine plus SCI when compared to the others (Figure 2C). There was an attempt to suggest that acute exposition to 3 days of 10 mM nicotine concentration was not enough to injure the tissue by itself, but a worse follow-up was defined for the SCI.

3.2 Nicotine raised a marked spinal cell death in response to ex-vivo SCI.

Spinal cord tissue slices were successfully cultured for up to a 2-week period. During this time, slices preserved their morphological and structural integrity with a clear differentiation of white and gray matter (Figure 2A).

Based on these macroscopic parameters, the MTT trial was carried out on the 14th day of the experiment, after nicotine exposure and SCI injury in the different groups. Half of the medullary tissue composed by both white and gray matter was analyzed under light microscopy and the purple precipitate that accumulated within the cytoplasm of living cells could be visualized (Figure 3A). At the same time, the quantification set of histograms (Figure 3B) was obtained by inverted microscopy which revealed a smaller number of viable cells on tissues which were acutely submitted to nicotine (for 3 days, at 10 mM concentration) and then suffered SCI, when compared to other groups. This lower viability was also confirmed by the MTT analysis under the absorbance of tissue cells by spectrometry (Figure 3C).

For DAPI of a medullary section, the authors analyzed the gray matter, also known as medullary "H", since it is the region which contains a higher concentration of nucleated cells. After 14 days of experimental protocol, nuclear morphological features were assessed using DAPI. As shown in figure 4A-B, there were no significant diferences between the four groups. All cells analyzed presented a regular round shape and a well-defined nuclear surface, and did not present morphological signs of nuclear irregularities by morphometric analysis. Nevertheless, the nicotine-treated group was the only one to have cells with morphological nuclear disturbances, that might be related to several mechanisms that affect cell survival processes. As demonstrated in Figure 4C, the cells probably maintained viability and resisted due to a short exposure period to nicotine, although they presented greater morphological signs of apoptosis with disintegrated nuclei which is due to the presence of cytoplasmic blebs (bubble formation).

Finally, cell death was compared in four groups of spinal cord tissue slices, where the green channel depicts live cells and the red channel depicts compromised/dead cells. The fluorescence (Figure 5A) analysis added to the merged pictures (Figure 5B) showed that

tissue slices exposed to nicotine followed by SCI showed higher death rates in resident spinal cord than the other groups analyzed. (Figure 5AB), in agreement with the MTT assay. The results found in this study also demonstrated that nicotine applied acutely, during the 3-day period, does not interfere in tissue recovery. In contrast, when the compound was administered before SCI, it hindered cell proliferation and the subsequent tissue regeneration, inhibiting spinal healing. It is important to emphasize that the capability to isolate viable cells after 14 days in *ex-vivo* culture also has demonstrated tissue viability until the end of the experimental protocol.

4. Discussion

Although several studies have shown the neuroprotective effects of nicotine (Badanavalu and Srivatsan, 2019; Kaur et al., 2018; Wang et al., 2019), the presence of this substance in the bloodstream is related to a series of deleterious events (Akça et al., 2020; Sananta et al., 2019). Necrosis is the most challenging of them and it occurs due to vaconstriction and reduced tissue perfusion (Tsutakawa et al., 2009). There are no studies analyzing the impact of nicotine on spinal tissue (Schweitzer et al., 2015; Scott et al., 2018; Tsutakawa et al., 2009). Thus, we seek to identify the repercussions of the use of nicotine after SCI in the spinal cord from adult rats by modeling traumatic injury *ex-vivo.*

In recent years, many studies have tried to evaluate the effectiveness of new drugs with a potential to reduce secondary damage from SCI and promote recovery. The neuroprotective effects of valproic acid in the preservation of motor neurons after SCI was evaluated by Pandamooz et al in an *ex-vivo* study model, in which valproic acid was administered one hour after the trauma, showing a significant reduction in cell death and preserving neural integrity, especially of motoneurons, which can mean a potential for valproic acid in further studies on motor improvement in patients with SCI (Pandamooz et al., 2017).

Considering the complex mechanisms of SCI, recent studies have been developed to elucidate this pathology in order to define the best model to be used, and possible therapeutic screens for spinal cord recovery (Guijarro-Belmar et al., 2019; Pandamooz et al., 2016; Pandamooz et al., 2019; Patar et al., 2019a). The use of spinal cord slice culture has several advantages compared to other models of spinal cord evaluation, as it allows the direct and real-time visualization of cell activity after the intervention and is effective in assessing cell viability, having been used previously in several other studies. Furthermore, the *ex-vivo* models of SCI fulfill the *3R concept* of animal use in SCI research models, which are replacement, reduction and refinement (Patar et al., 2019b). Thus, this model is a great way to analyze the effect of pharmacological agents on spinal trauma, before moving to larger studies involving animals. It is important to emphasize that the use of rats as the

experimental model of SCI has well-structured protocols that are widespread in several research groups, however, it has required intensive care after post-operative time, higher investiment and normally they are more time-consuming.

Ex-vivo models of spinal cord injury optimise novel interventions and mimic *in vivo* injury before preclinical studies (Guijarro-Belmar et al., 2019; Patar et al., 2020). Here we described an *ex-vivo* adult rat model of SCI for testing the effects of nicotine on spine tissue, after an SCI. Furthermore, the model presented in this study allowed us to investigate the impact of nicotine on tissue, maintaining cellular interactions and their mechanisms in a multicellular environment *in vitro*, using a fast and relatively simple platform, which retains the architecture of the tissue and preserves its neural activities.

In this study, cultured slices of adult rats were subjected to a contusive model of SCI. The process of dissecting, slicing and in vitro culturing for 4 days was performed. Then, spinal cord injury was applied to the slices by dropping a 0.5 g weight from a 25 mm height. After the assessment of nuclear morphology, tissue viability and vitality, it was found that cells exposed to nicotine and trauma showed morphological changes, such as an increase in the number of apoptoses, as well as less viability, when compared to control cells or the groups that received the interventions alone. This fact may be explained because the presence of nicotine in the bloodstream is correlated with an increased level of tissue necrosis, impairing the healing process (Falavigna et al., 2013; Hillard et al., 2007).

Nuclear morphology and cell viability were evaluated by the DAPI which consists of a DNA-specific probe that attaches to the minor groove of A-T sequences of DNA, forming a fluorescent complex (Banerjee and Pal, 2008; Chazotte, 2011; Kapuscinski, 1995). As shown in the results section, there were no statistically significant diferences between groups exposed and not exposed to nicotine and SCI. On the other hand, despite not being statistical, apoptosis was more evident in the nicotine exposed group.

However, the authors are aware that *ex-vivo* models may have a number of limitations, and cannot replace all *in vivo* studies. As a disadvantage, an *ex-vivo* model cannot be used to monitor functional recovery, nor do they have the intact blood supply of the

in vivo model systems (Pandamooz et al., 2016; Patar et al., 2019a; Patar et al., 2019b). *Ex-vivo* systems do not allow accessing immune cells circulating in the bloodstream, which infiltrate the damaged tissue, such as monocytes and macrophages, since the slice culture system lacks a blood supply. This model also assessed short-term changes of spinal tissue after an SCI (5 days), whereas spinal trauma in humans might present complex and dynamic changes over longer periods of time. An advantage for our study was that it used slices derived from adult rat spinal cord, which are previous known to have less neuronal survival when compared to newborn rat tissues (Bonnici and Kapfhammer, 2008; Falavigna et al., 2013; Patar et al., 2020; Pohland et al., 2015).

According to previous hypotheses, it was concluded that exposure to nicotine harms the viability and cell survival of injured spinal tissue. Given that nicotine is widely consumed worldwide, and spinal trauma is a possibly limiting condition, it is important to establish the relationship between nicotine and traumatic SCI, in order to demonstrate that this substance worsens the prognosis of patients who suffer from TRM. Studies are in progress in the research group to apply a stain for cell differentiation and electron microscopy and elucidate *in vivo* mechanisms and therapeutic protocols that could assist in the healing process of injured spine, since this therapy has the potential to repair capillary angiogenesis, thus favoring cell proliferation and tissue restoration.

5. Acknowledgments

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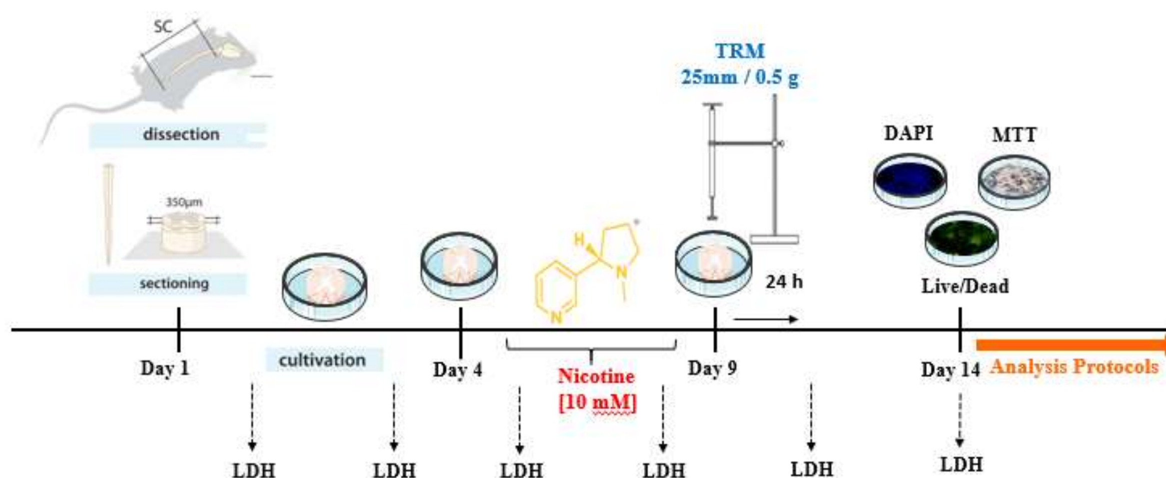

Figure 1. Schematic study design. Day 1: The animals were euthanized, the spinal cord was removed, and the spinal slices were made. Day 4: After the tissue remained in culture for 4 days, the protocol for exposure to nicotine (10mM) was initiated and lasted for 3 days. Day 9: SCI was performed through the use of Impactor, with 5g in the pendulum and a drop height of 25mm, which caused a moderate spinal cord injury. Day 14: Cell Viability with MTT, Nuclear Morphology and Morphometric Analysis by DAPI and Live/ Dead Cell Viability tests were performed. Culture supernatant collections for analysis of de Lactate Dehydrogenase Activity were collected on days 1, 3, 5, 8, 10 and 14.

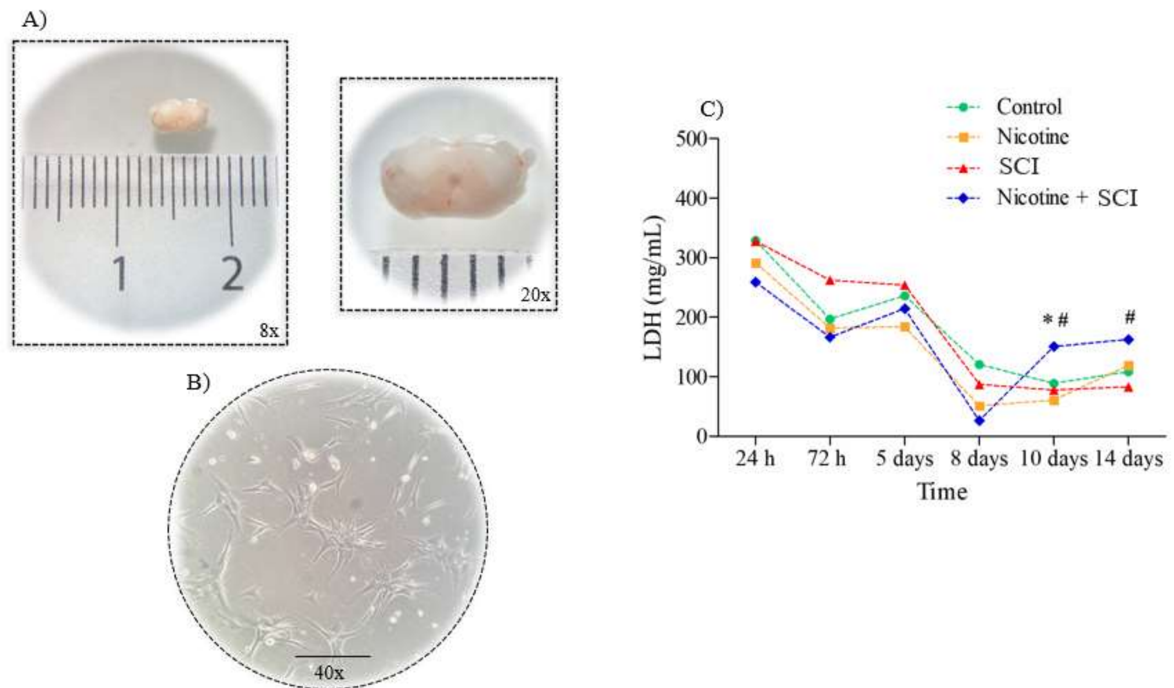

Figure 2. Evolution of the *ex-vivo* model and LDH activity. A) Representative images of the evaluation of the medullary slice with a stereoscopic magnifying glass (8x and 20x of magnification). B) Isolated spinal cord resident cells from the culture supernatant. Optical microscopy (40x of magnification). C) Evolution of culture's LDH activity during the experiment.

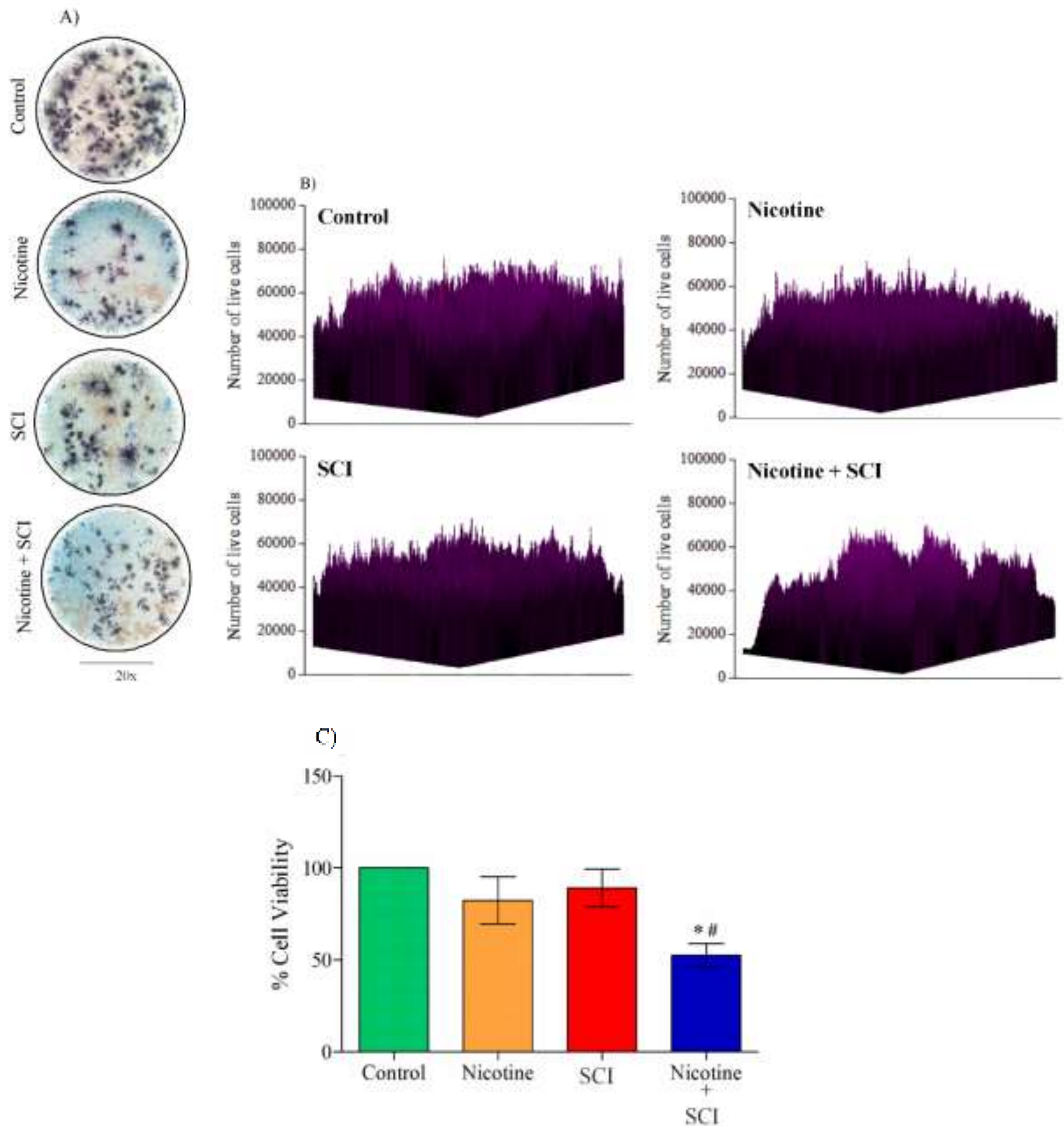

Figure 3. Cell Viability Tissue Assay. A) Representative images of Control, Nicotine, SCI and Nicotine-SCI groups, respectively. We observed a significant reduction in cells residing in the marrow in the Nicotine-SCI group. Optical microscopy (20x). B) Quantification of cell viability illustrated by the histogram, showing its reduction in the Nicotine-SCI group. C) Analysis of absorbance by spectrometry, which corroborated with previous data, represented by the graph.

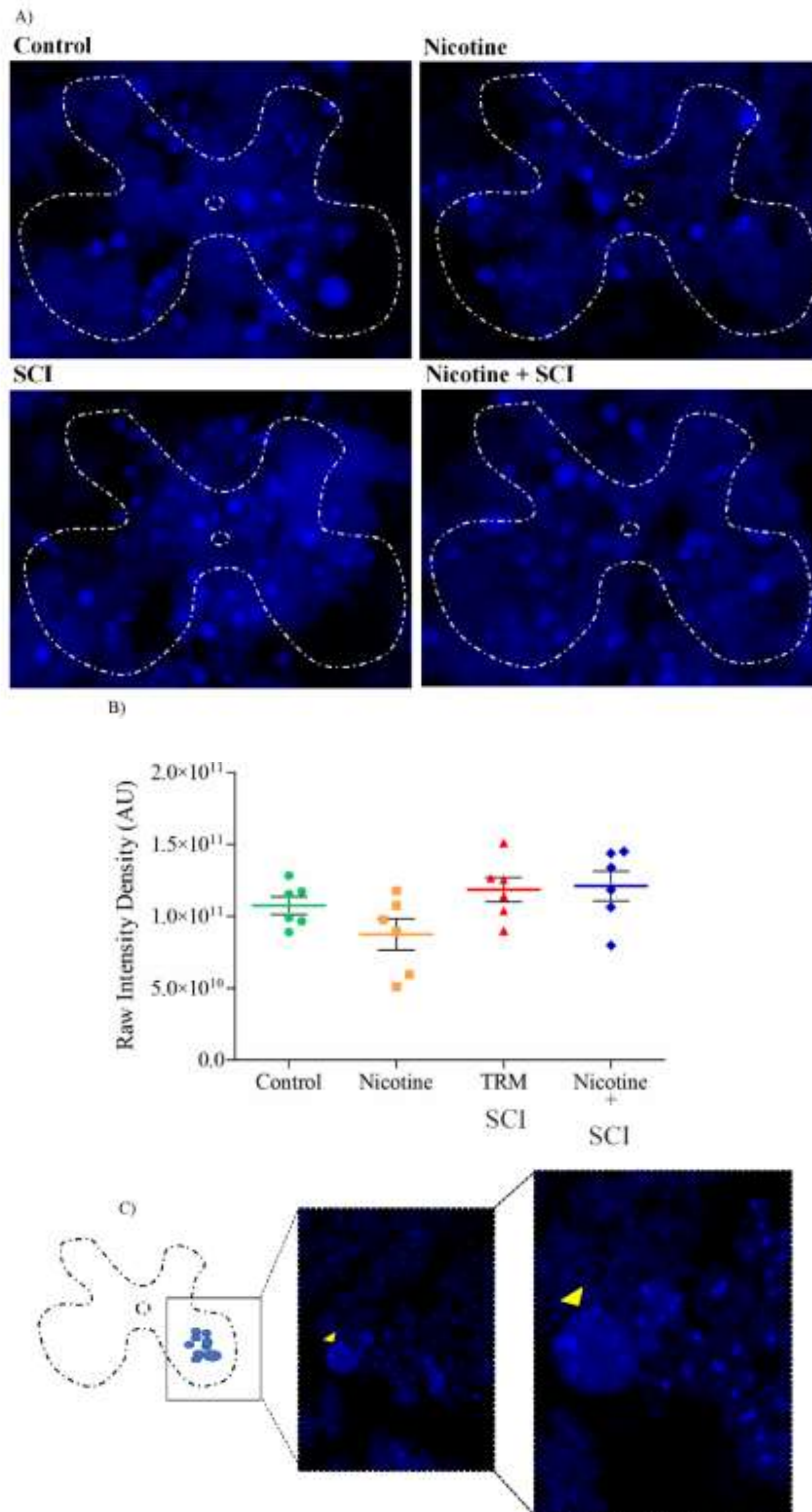

Figure 4. Nuclear Morphology and Morphometric Analysis by DAPI. A) and B) Analysis of the gray matter, after 14 days of experiment, represented by images and graph, demonstrating that there were no significant differences between the groups. SEM (20x). C) Illustration of apoptosis, presence of disintegrated nuclei and SEM cytoplasmic bubbles (20 and 40x).

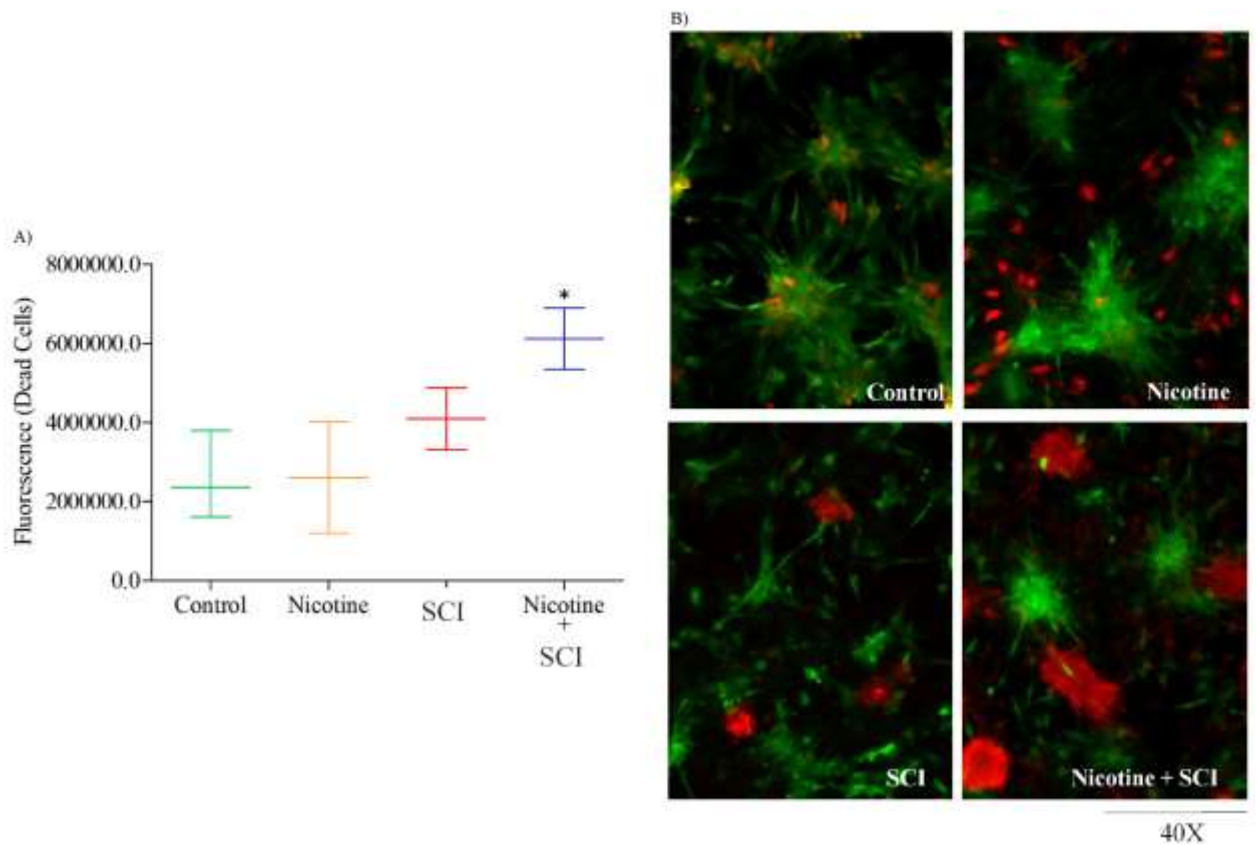

Figure 5. Analysis of Live/Dead Cells. A) The graph shows that the Nicotine + SCI group had higher rates of cell death than the other analyzed groups. B) Illustrative images evidencing that Nicotine + SCI group colored the reddish color more than the others, emphasizing cell death / damage.

4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Frente aos diversos estudos que colocam a nicotina como protagonista na terapia de regeneração de diversos sítios celulares, principalmente àqueles ligados ao sistema nervoso central, o modelo *ex-vivo* proposto no presente trabalho evidenciou que a exposição do tecido medular à nicotina, com subsequente lesão por impacto, diminui a viabilidade e a sobrevivência celular, o que diretamente interfere no processo de reestruturação tecidual e consequente cicatrização.

Embora a exposição isolada do tecido medular ao TRM ou à nicotina, não interferiram de forma significativa na recuparação da viabilidade e sobrevivência celular, a análise da associação de ambos os eventos - exposição à nicotina seguido de lesão tecidual por TRM, sugere um impacto negativo da nicotina na recuperação do tecido medular, provavelmente ligado ao ambiente hipóxico que esta exposição ocasiona. É importante ressaltar que o presente modelo avaliou a exposição aguda à nicotina, por 3 dias consecutivos, o que pode ser a razão de os potenciais efeitos deletérios deste composto não terem sido percebidos quando de forma isolada.

Em virtude da escassez de trabalhos correlacionando a nicotina com o TRM e a nicotina com o tecido medular, é importante que mais investigações sejam realizadas, a fim de elucidar essa relação e possibilitar a aplicação de protocolos terapêuticos factíveis.

O modelo experimental apresentado proporciona a utilização de profusas formas de investigação sobre o mecanismo do TRM e possíveis triagens terapêuticas. Estudos estão em andamento no grupo de pesquisa a fim de caracterizar as populações celulares atingidas e/ou preservadas em presença da nicotina e submetidas ao TRM. Neste sentido, a utilização de modelo de TRM *in vivo* surge como a perspectiva que nos permitirá a avaliação funcional da regeneração do tecido medular, a fim de aprimorar protolocos e macanismos terapêuticos que podem favorecer a cicatrização medular.

5 ANEXOS

5.1 Confirmação de submissão

Experimental Neurology

Acute nicotine exposure evokes spinal cord cell death in an ex-vivo traumatic spinal cord injury model

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	Neurological Disorders: CNS Trauma
Keywords:	Nicotine; spinal cord injury; cell death; ex-vivo model
Corresponding Author:	Natália Fontana Nicoletti, PhD University of Caxias do Sul: Universidade de Caxias do Sul BRAZIL
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	João Pedro Einsfeld Britz
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	Dr Asdrubal Falavigna, MD, PhD
Abstract:	Nicotine is a psychoactive drug that has deleterious effects. However, there is little literature concerning its effects on spinal tissue. Spinal cord injury (SCI) resulting from spinal lesions that cause changes in sensitivity, motor and autonomic neuronal function, which can lead to irreversible life-changing neurological damage. Nicotine has been linked to increased tissue necrosis, resulting from vasoconstriction and decreased local perfusion. In order to understand this relation, this work investigated the effect of nicotine on spinal tissue after SCI, using the ex-vivo model of a three-dimensional culture of spinal tissue from Wistar rats. The tissues were incubated for 3 days, followed by the protocols of nicotine induction (10 mM for 3 days acute exposition), and SCI compression by impact from a height of 25 mm and weight of 0.5 grams in the center of the medullary tissue section and then 14 days of follow-up. Samples of the supernatant were collected at 24h up to 14 days for LDH analysis. By the end of the experiment, MTT tests, nuclear morphology by DAPI and LiveDead cell assays were performed. The tissues that were exposed to nicotine and then suffered SCI had less cell viability and showed higher levels of LDH, when compared to the other groups. Since SCI is a limiting condition and nicotine is a potentially vasoactive substance, it was possible to assess its effects on medullary tissue and verify that both hampered tissue recovery. This study has important implications and encourages the elucidation of the nicotine mechanism in SCI.
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	Mohammed Akmal M.Akmal@orthopaedics.com
	Cheryl A Lane clane@uabmc.edu

5.2 Carta de Aprovação Comissão de Ética no Uso de Animais – UCS

UNIVERSIDADE DE CAXIAS DO SUL
COMISSÃO DE ÉTICA NO USO DE ANIMAIS
CEUA-UCS

Caxias do Sul, 30 de agosto de 2018.

Of.015/2018-CEUA

Número: 014/2018

Título: "Efeitos da Nicotina na Lesão Medular Traumática Associada ao Tratamento com Oxigeno-terapia Hiperbárica".

Investigadores Principais: Prof. Asdrubal Falavigna

A Comissão de Ética no Uso de Animais da Universidade de Caxias do Sul, em reunião ordinária do dia 24 de agosto de 2018, analisou o projeto supracitado e o considerou **aprovado**, de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA).

FINALIDADE	( ) ENSINO (X) PESQUISA CIENTÍFICA
Vigência da autorização	08/2020
Espécie / linhagem / raça	Ratos Wistar
No. de animais	58
Peso / Idade	250g / 14 semanas
Sexo	Fêmeas
Origem	CREAL-UFRGS

Obs.: Este ofício substitui o Of. CEUA 028/2017 / Número 021/2017.

Atenciosamente,

Prof. Dr. Matheus Parmegiani Jahn
Coordenador CEUA/UCS

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