André de Oliveira Arruda

NANOFIBRAS POLIMÉRICAS POR TÉCNICA DE FIAÇÃO POR SOPRO EM SOLUÇÃO – INOVAÇÃO EXPERIMENTAL COMO PROTÓTIPO EM CONTEXTO TERAPÊUTICO DO TRAUMA RAQUIMEDULAR

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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Caxias do Sul

2020

Dados Internacionais de Catalogação na Publicação (CIP)
Universidade de Caxias do Sul
Sistema de Bibliotecas UCS - Processamento Técnico

A779n Arruda, André de Oliveira

Nanofibras poliméricas por técnica de fiação por sopro em solução – inovação experimental como protótipo em contexto terapêutico do trauma raquimedular [recurso eletrônico] / André de Oliveira Arruda. – 2020.

Dados eletrônicos.

Dissertação (Mestrado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Ciências da Saúde, 2020.

Orientação: Asdrubal Falavigna.

Modo de acesso: World Wide Web

Disponível em: https://repositorio.ucs.br

1. Coluna vertebral - Ferimentos e lesões. 2. Materiais biomédicos. 3. Nanofibras. 4. Medicina regenerativa. I. Falavigna, Asdrubal, orient. II. Título.

CDU 2. ed.: 616.711-001

Catalogação na fonte elaborada pela(o) bibliotecária(o)
Ana Guimarães Pereira - CRB 10/1460

UNIVERSIDADE DE CAXIAS DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

COORDENADOR DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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Dedicatória

Dedico essa dissertação primeiramente a Deus, em seguida aos meus pais, Laerte Arruda e Djanira de Oliveira Arruda, familiares, amigos e demais incentivadores.

Agradecimentos

Aos meus mentores, agradeço o trabalho conjunto, correções e oportunidade de crescimento profissional e pessoal.

Ao Professor Doutor Asdrubal Falavigna, pela amizade, oportunidade de desenvolvimento, orientação, estímulo, concepção e execução do projeto, bem como recepção em Caxias do Sul, junto à sua família.

À Professora Doutora Natália Fontana Nicoletti Roxo, pela colaboração quanto à concepção do projeto e execução dos estudos celulares e correlacionados no Laboratório de Transplante Celular da Universidade de Caxias do Sul.

À Professora Doutora Fernanda Trindade Gonzalez Dias, pela colaboração quanto à concepção do projeto e execução dos estudos preliminares e de aplicação correlacionados nos Laboratórios da Escola de Engenharia de Materiais da Universidade de Caxias do Sul.

Ao Professor Doutor Otávio Bianchi, pela colaboração quanto à concepção do projeto.

Aos alunos da Graduação e Pós-Graduação, Leonardo Galli Engler e Lucas Dall Agnol, pela execução dos testes relacionados à manipulação de materiais nos Laboratórios da Universidade de Caxias do Sul.

Aos amigos André Moreira Castilho e Vitor Viana Bonan de Aguiar, pela amizade, incentivo mútuo e jornada conjunta.

Ao Professor Mestre Emiliano Neves Vialle, pela amizade, apoio, incentivo e oferecimento de oportunidades de participação e aprendizagem em pesquisa científica.

À Instituição AOSpine Latin America, pelo apoio e incentivo à pesquisa em cirurgia da coluna por meio de disponibilização de bolsa de estudos integral para Programa de Mestrado em Ciências da Saúde da Universidade de Caxias do Sul.

Sumário

1 INTRODUÇÃO

O trauma raquimedular (TRM) corresponde a um evento delimitado e bem definido, de etiologia traumática em sua maioria e que exerce efeito drástico nos pacientes, uma vez que leva à disfunção motora e sensorial permanentes (1). Imediatamente após o acontecimento da lesão mecânica (lesão primária), uma sequência complexa e em cascata de eventos é automaticamente deflagrada, resultando, em conjunto, no que é definido como lesão secundária. Essa compreende cadeias de reações deletérias ao tecido neural, como morte de neurônios e oligodendrócitos, desmielinização neuronal, gliose, inflamação, apoptose e necrose (2), gerando desafios múltiplos para uma estratégia terapêutica eficiente, devido à sua alta complexidade. Até o momento, as estratégias terapêuticas para o TRM têm se concentrado principalmente na fixação – estabilização da coluna (cirúrgica), combinada ou não com alternativas potenciais, mas ainda não efetivas, como medicamentos (corticosteroides e outros), fatores neurotróficos e transplante de células-tronco (1) – além do manejo clínico de elevada complexidade. Ainda existem muitas barreiras para bons resultados clínicos e, possivelmente, em etapas futuras, regeneração do tecido neural, com expressão de melhora clínica. Há que se considerar que a abordagem cirúrgica não induz nenhum efeito sobre a regeneração tecidual, vários são os efeitos colaterais críticos das drogas, ocorre uma degradação rápida do fatores neurotróficos aplicados e ainda muitos desafios para a terapia por células-tronco, incluindo a possibilidade de geração de pressão no tecido local após a implantação dessas, agravando as condições locais e piorando a lesão (1). Dito isso, no que diz respeito à medicina regenerativa, a engenharia de polímeros – macromoléculas formadas pela união de substâncias simples, chamadas monômeros – mostra-se promissora, pois é capaz de atuar na reconstrução de tecidos, reduzindo a formação de cicatrizes gliais e controlando os processos de lesão secundária no contexto do TRM (1).

Os biomateriais implantados são capazes de reparar ou substituir a função e a estrutura anatômica dos tecidos danificados por meio das características morfológicas dos polímeros e fibras fabricadas por diferentes metodologias (3). Estudos prévios já demonstraram o potencial de reparo do sistema nervoso central (SNC), aplicando polímeros naturais ou sintéticos (4), uma vez que são capazes de mimetizar muitas características do ambiente extracelular nativo, podendo também ser utilizados como

veículo de liberação de agentes terapêuticos localmente (5, 6). Todos os biomateriais destinados à aplicação em ambiente tecidual vivo precisam ser polímeros altamente adaptáveis, possibilitando que sejam projetados para atingir propriedades topográficas e físico-químicas que podem simular os microambientes nativos necessários para a regulação da função celular de destino (7). Além disso, o material deve ser biocompatível em nível suficiente para não elicitar resposta imune do hospedeiro (8), ou, ao menos, se impossível tal condição, gerar reação local passível de controle.

Há uma ampla gama de aplicações médicas de polímeros como nanofibras, variando de andaimes para regeneração de tecidos a sistemas de distribuição de drogas (9). As nanofibras de polímero precisam ser integradas ao tecido vivo nativo, simulando as características da matriz extracelular (ECM) e permitindo a sobrevivência e o desenvolvimento celular (10). Já as propriedades finais das fibras - alinhamento x anisotropia, tamanhos de poros e fibras, emaranhamento, taxa de degradação e outras - são cruciais para resultados favoráveis e variam muito, considerando diferenças naturais entre alvos - como especificações de nervos e tecidos da medula espinhal. Os requisitos fundamentais para a aplicação de polímero em tecidos vivos são necessariamente alta porosidade, juntamente com uma alta proporção de área de superfície para volume, permitindo a infiltração celular, troca de gás e nutrientes e maximizando as interações da superfície da célula, criando um ambiente biomimético ideal (11, 12).

Uma recente técnica de manipulação de materiais chamada "*Solution Blow Spinning*" (SBS) – Fiação por Sopro em Solução, pode produzir nanofibras utilizando uma ampla variedade de polímeros, com elevada taxa de fabricação / fiação, em comparação com as técnicas tradicionais, como a eletrofiação. O sistema SBS consiste em agulhas concêntricas, por meio das quais uma solução de polímero e um gás pressurizado são ejetados simultaneamente. Pelo orifício de saída da agulha, aproveitando-se do arrasto aerodinâmico e das forças de cisalhamento causadas pelo fluxo de gás pressurizado, auxiliando também na evaporação do solvente, ocorre a produção e saída das nanofibras secas (13) – em combinação, para formar um cone como o cone de Taylor (14). Mais seguro e simples do que a eletrofiação, permitindo também a fiação de modo mais rápido e mostrando versatilidade na escolha do solvente (13), a técnica SBS permite a deposição de fibras diretamente sobre qualquer tipo de superfície, incluindo sistemas vivos, como no caso da constituição de enxertos

de suporte, selantes ou tecidos adesivos (13, 15). Dessa forma, por meio desse sistema, seria possível a cobertura por nanofibras em uma ampla gama de alvos (16), potencialmente sem limitações para lesões medulares, considerando que a lesão primária determina um defeito local (mesmo que por compressão), que pode ser, por sua vez, afetado positivamente pela deposição local de nanofibras.

Em contrapartida, cabe destacar que vários são os obstáculos atuais em relação à aplicação translacional. Como exemplos, destaca-se inicialmente que os diferentes métodos de engenharia de tecidos variam quanto à fabricação e manipulação da fibra; em seguida, tem-se que os pré-requisitos para a produção de nanofibras são, em geral, incompatíveis com a biologia e metabolismo nativos locais – como a necessidade obrigatória de eletricidade e coletor carregado (método de eletrofiação) (17) ou ainda a utilização de solvente orgânico, prejudicando a retenção da atividade do polímero biológico (18), além de afetar negativamente o tecido local, havendo significativo potencial de remanescente no produto final. A maioria dos estudos da técnica SBS aplica solventes orgânicos altamente voláteis ou misturas com esses, tendo o uso de opções menos voláteis – como água – como um desafio crítico (16, 19) em combinação com a proposta de aplicação segura em tecidos vivos, complexos e sensíveis – como o sistema nervoso central (SNC).

O polímero escolhido corresponde a outro ponto crítico para a engenharia de tecidos em contexto biomédico, havendo várias características consideradas como obrigatórias, favoráveis e impeditivas de aplicação em tecido nativo, intrínsecas aos materiais – exigindo o estabelecimento da combinação otimizada dessas. O poli (álcool vinílico) - PVA, obtido a partir da hidrólise controlada do poli (vinilacetato) (PVAc), apresenta vantagens consideráveis sobre demais polímeros, cabendo destaque: sua solubilidade em água, não toxicidade a tecidos e diversas linhagens celulares e boas estabilidades química e térmica (19, 20), mostrando-se um candidato promissor para estudos de seguimento translacionais. Tem-se registros de que o PVA foi testado em diferentes contextos – cartilagem artificial (21), disco intervertebral (22), osso (23), tecidos vasculares (24) e para a liberação controlada de substâncias ativas (25) e curativos (26) – incluindo-se o SNC como destino final, havendo demonstrado efeito positivo, sendo capaz de diminuir a reação inflamatória local e permitir que a medula espinhal e raízes nervosas pudessem manter seus respectivos movimentos normais, sem aderências (27).

Considerando uma possível etapa posterior de translação, a interação entre nanofibras poliméricas e células precisa ser aceitável, havendo clara necessidade de investigações que abordem tais interações – análise de morfologia celular, adesão, proliferação e diferenciação celular (28, 29), além de estudos funcionais. O sucesso biológico e metabólico final de novas terapias médicas depende de como as células respondem aos biomateriais nanoestruturados obtidos (9).

Sendo assim, frente ao contexto apresentado, este estudo visa, de modo inédito a desenvolver e determinar os parâmetros de produção de nanofibras por SBS, envolvendo o aparato e a solução polimérica, além da interação das nanofibras com as células, aplicando PVA em solução aquosa como um protótipo com potencial translacional para composição de estratégia terapêutica em contexto de TRM.

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3 ARTIGO

POLYMER NANOFIBERS PRODUCED BY SOLUTION BLOW SPINNING METHOD – AN INNOVATIVE EXPERIMENTAL PROTOTYPE TOOL TO SPINAL CORD INJURY

1. INTRODUCTION

Spinal cord injury is a well-defined and usually traumatic condition that has drastic effects, leading to permanent motor and sensory dysfunction (1). A sequence of events is started after a mechanical injury, resulting in the secondary damage – neuronal demyelination, gliosis, inflammation, apoptosis, and necrosis (2), and generating challenges for an effective therapy. Treatments for SCI are focused on spinal stabilization (surgical), combined, or not, with drugs, neurotrophic factors and stem cell transplantation (1), besides critical clinical management. Unfortunately, there are meaningful side-effects of drugs, fast neurotrophic factor degradation, and challenges for stem cell therapy – including the possibility of pressure generation on local tissue after implantation, worsening local conditions and lesion (1).

Material engineering in regenerative medicine by polymer application is promising as it can act on tissue reconstruction, reducing the formation of glial scars and controlling the secondary lesion processes (1). Biomaterial implants repair or replace the function and anatomical structure of impaired tissues through polymers' morphological characteristics and fabricated fibers (3). Studies have demonstrated the potential for central nervous system (CNS) repair, applying natural or synthetic polymers (4), since they can mimic features of the extracellular native environment, and be used as a vehicle to release therapeutic agents locally (5, 6). The biomaterial must be biocompatible enough not to elicit host immune response (8), or, at least, if impossible, a controlled local reaction. There is a wide range of medical applications of polymeric nanofibers (9), having them integrated to native live tissue, simulating extracellular matrix (ECM) characteristics and allowing cell survival and development (10). Since the final properties of fibers – alignment, diameter, entanglement, degradation rate – are crucial for favorable results, the differences between targets need to be considered. Fundamental requirements for application on live tissues are necessarily as a high porosity along with a high surface area to volume ratio, allowing cellular infiltration, gas and nutrient exchange and maximizing cell-surface interactions, creating an ideally biomimetic environment (11, 12).

An innovative technique named “solution blow spinning” (SBS) can produce polymer micro/nanofibers at a higher fiber output. The SBS system consists of concentric nozzles through which a polymer solution and a pressurized gas are simultaneously ejected (13, 14). Safer and simpler than electrospinning (17), SBS enables the deposition of fibers directly onto any surface, including living systems (13, 15), “painting” nanofibers onto a broader range of targets (16), with potentially no limitations to medullary lesions. Instead, there are many obstacles to a translational application, as the usage of an organic solvent, making it difficult to load and retain biological polymer activity (18), in addition to harming local tissue by potential remnants. Most of the SBS studies use highly volatile organic solvents or mixtures, and the use of less volatile ones, as water, is a great challenge (16, 19) considering safe application on complex and sensitive tissues – central nervous system (CNS).

The polymer chosen in the biomaterial field is critical. The poly(vinyl alcohol) – PVA, has positive characteristics: water solubility, nontoxicity, and good chemical and thermal stability (19, 20). PVA was tested in different contexts – artificial cartilage (21), intervertebral disc (22), bone (23), and vascular (24) tissues, controlled release of active substances (25) and wound dressing (26) – including CNS, demonstrating a positive effect capable of decrease the inflammatory local reaction (27). New medical therapies' ultimate functional success depends on how cells respond to the nanostructured biomaterials obtained (9), needing studies and analysis of cell morphology, adhesion, proliferation, and differentiation (28), besides functional tests.

This project aims to investigate the potential of blow spun PVA nanofibers produced from aqueous solutions as a translational prototype for SCI therapeutic strategy approach – fiber fabrication method feasibility and cellular interaction. Although PVA-based nanofibers have already been used in

other biomedical applications, this is the first time in the literature that such systems will be tested in medullary tissue and SCI environment.

2. MATERIALS & METHODS

2.1. Chemicals & Polymer solution

Table 1 shows the materials used in the experiments to prepare polymer solutions – all directly tested, non-purified or processed again before tests.

Polymer	Details	Solvent	Concentration
PVA 1	104500 $g.mol^{-1}$ molar mass, Neon Comercial (São Paulo, Brazil)	Milli-Q H_2O	1 to 20% (w/w)
PVA 2	89000 – 98000 $g.mol^{-1}$ molar mass, Sigma-Aldrich (São Paulo, Brazil)	Milli-Q H_2O	1 to 20% (w/w)
PVA 3	31000 – 50000 $g.mol^{-1}$ molar mass, Sigma-Aldrich (São Paulo, Brazil)	Milli-Q H_2O	15% (w/w)

Table 1: Polymer solutions used in the experiment

Three different grades of PVAs were tested for an ideal solution, with the fibers' spinnability and diameter as the main parameters for the systems' performance. PVA/water solutions with concentrations between 1–20% (w/w) were initially tested to evaluate the systems' solubility and viscosity. Solutions were prepared at different solubilization temperatures and times. The solutions were then transferred to 25 mL syringes and examined for fiber formation in a laboratory.

2.2. Solution blow spinning apparatus

Authors designed the SBS equipment applied to this project, using a commercial airbrush (Steula, BC 61) with different inner nozzle diameters – 0.2, 0.3 and 0.5 mm – fixed in a support with controlled lateral movement, connected to an air compressed lab air system with a pressure control varying from 1–7 bars. A simple collector was positioned at different distances from the SBS's external nozzle, which is also a crucial parameter for fiber's formation and quality. A 2000 W thermal blower was incorporated into the SBS system, under 30° of inclination to the nozzle, 12 m/s airflow, and 120° C maximum temperature at the line, to assist in solvent evaporation during spinning (Fig. 1).

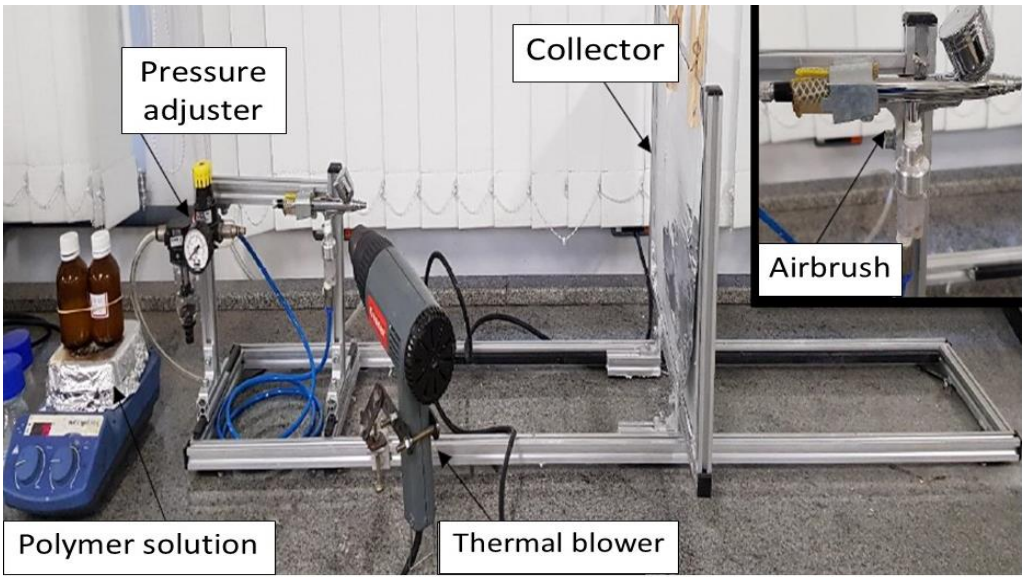


Fig. 1: SBS apparatus – airbrush on detail (upper right corner)

2.3. Solution & fibers characterization

Prepared PVA solutions were evaluated by rheology (Anton Paar rheometer, MCR 301, 50 °C – data not shown) as to the critical concentration (c*) for obtaining fibers since SBS's fiber formation needs a sufficient entanglement by polymer chains. Polymer solutions prepared at concentrations below c* for a particular polymer-solvent system do not form fibers. Fiber morphologies were examined by Field Emission Gun Scanning Electron Microscopy (FEG-SEM Mira 3 Tescan, 10 kV magnification images) to determine their average diameter – a parameter that directly interferes with the biomaterial implant's surface and biological recognition properties

2.4. Cell lineages and general cell culture protocols

Different cell lineages were tested in terms of interaction with PVA polymeric nanofibers fabricated by the SBS method, as follows VERO (kidney epithelial cells of the African green monkey, Sigma Aldrich), 3T3 (murine fibroblast, Sigma Aldrich) – standardized previously for cytotoxicity and polymers x cells interaction tests – and expanded resident spinal cord cells non-specific characterized from rats. To evaluate the interactions between cells and PVA nanofibers, an elution process of biomaterial was performed, incubating the cell lines in a biomaterial solution at a previously eluted size corresponding to 3 cm^2 / mL, as recommended by ISO 10993 (30), under aseptic conditions, in a class II safety cabinet. The cell lines were seeded at 3-5×10^3 cells per well in 96-well plates, or 15-20×10^3 cells per well in 24-well plates, depending on the experimental protocol. All experiments were performed three times in triplicate with cell culture passages P2-P3.

2.5. Cell viability

In order to assess cell viability, the VERO and 3T3 lineages, were evaluated by their metabolically active mitochondria using MTT assay. The cell lines were incubated with PVA 2 18% nanofibrous SBS fabricated mats (N=3 per group per experiment) extracted from the collector and eluted at 24 hours, 48 hours, 72 hours, and 7 days – all experiments were performed three times in triplicate.

2.6 Nuclear morphology and mitotic index

The 40,6-diamino-2-phenylindole (DAPI) staining was carried out to establish the nuclear morphology of the tested cells – seeded in 24-well plates and incubated with fixed PVA 2 18% nanofiber elution for 24, 48, 72 hours and 7 days. After incubation, the cells were washed three times in PBS, and fixed with 4% formaldehyde at room temperature, for 15 min. The fixed cells were then washed with PBS, permeabilized with 0.1% Triton X-100 in PBS and stained with a 300 nM DAPI solution (Santa Cruz, CA) at room temperature, for 10 min. The nuclear morphology of the cells was examined under a fluorescent microscope (Carl Zeiss Micro Imaging GmbH, Germany). DAPI staining delineates mitotic figures and enables mitotic index determination for each automatic cell count. The mitotic index was calculated as the number of mitotic events in 10 fields per well, three times in triplicate. DAPI staining clearly delineates the nuclear morphology that allows the quantification of the nuclear roundness and solidity measurements by Image J Software. Data from control cells (untreated) are used to set the parameters of the normal population. The morphometric parameters were calculated considering 100 events, three times in triplicate.

2.7 LIVE/DEAD Assay

For the identification and quantification of dead cells in the VERO cell line and resident spinal cord cells, it was employed the LIVE/DEAD Cell Viability Assay kit (ABKINE). Cells were incubated for the cell labelling, following the manufacturer's specification. In this assay, we load cells with Calcein-AM plus propidium iodide (PI), which upon penetrate into live (green fluorophore) and dead (red fluorophore) VERO and resident spinal cord cells, respectively, and count red/green fluorescent cells. Then, the slices were placed back into de incubator for 30 min to allow permeation of Calcein-AM and PI. Acquisition images were taken immediately in the four regions of gray matter for each group, three

times in triplicate. The analysis of pictures and analyzes of the data were performed using an inverted microscope setup for fluorescence microscopy AXIOVERTII (Carl Zeiss MicroImaging GmbH, Germany) and its respective Zen Blue software.

2.8. Ex vivo animal model sample test

Considering the *ex vivo* SCI models (31, 32), to evaluate the interaction between direct PVA 2 18% nanofibers SBS fabricated over spinal cord tissue, a sample rat had its spinal cord extracted and adapted on collector apparatus. Afterwards, a surgical microscope showed the adhesiveness and water biodegradation over a time-lapse exposed interaction. Illustrative images were taken, considering translational studies (not for statistical purposes), and the sample was kept conditioned in humid chamber until 48 hours.

2.7. Statistical analysis

Data from *in vitro* experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, CA, U.S.A.). $P<0.05$ was indicative of statistical significance.

3. RESULTS

3.1. General results

All experiments involving animal usage and/or collection of live tissue were performed with Institutional Ethical Committee approval (CEUA 014-15/2018). The SBS apparatus with adjusted parameters allowed fiber production in nanoscale diameter, showing solution blow spinning as a feasible technique for polymer delivery to medullary tissue in a translational context.

3.2. SBS parameters & polymer solution

After a range of tests, critical points and optimized results – considering fiber formation, diameter distribution and range, solvent (Milli-Q water) evaporation and others – the following best parameters were: a 30-cm working distance (from outer nozzle to collector) allowed fiber deposition with good entanglement and no residual droplets; an outer nozzle diameter of 0.5 mm did not become clogged an allowed solution exit as a cone formation; gas pressure applied on a 7 bar system was able to drag the polymer solution smoothly and regularly; the concurrent use of 2 thermal blowers helped significantly with other solvent evaporation.

Regarding the polymer solution, after tests for different options (PVA 1 to 3) and concentrations, the ideal for fiber formation was defined as PVA 2 in 18% (w/w) concentration. The main tests and ideal result, considering fiber fabrication by SBS apparatus are summarized on Table 2.

Variable	Options	Best
PVA polymer grade	PVA 1 PVA 2 PVA 3	PVA 1: high viscosity solution, no fiber formation at the concentrations tested PVA 2: fiber formation, 18 % w/w best concentration tested PVA 3: insoluble in tested conditions
Gas pressure (bar)	1 – 7	7 (dragged the polymer solution smoothly and regularly)
Working distance (cm)	15 – 90	30
Outer nozzle diameter (mm)	0.2 0.3 0.5	0.5 (did not clog and allowed solution exit as cone conformation)
Thermal blower (number used)	1 2	2 (the solvent removal efficiency was better using two thermal blowers simultaneously)

Table 2: Parameters for best PVA nanofiber fabrication by SBS apparatus

3.3. PVA nanofibers

After PVA 2 was determined as the ideal polymer option, the nanofiber fabrication tests continued. As explained before, based on optical microscopic fiber images directly after SBS formation, electronic microscopic analysis followed. Selected FEG-SEM microscopy images are shown in Figure 2 to illustrate the influence of PVA 2 solution concentration (15%, 16%, 18% and 20%, w/w) on the diameter behavior of the fibers produced by SBS. Figure 2(a) shows the result for the 15% w/w PVA 2 solution spinning, where it is possible to observe remaining solvent droplets and fibers with 200 - 400 nm diameters. In Figure 2(b), fewer solvent droplets are seen for the blow-spun 16% w/w PVA 2 solution, but poor chain entanglement is denounced by fibers with smaller and more dispersed diameters. Figure 2(c) shows the fibers of better quality and the most uniform distribution of diameters (200 – 300 nm), making the 18% w/w PVA 2 solution the most suitable to be blow-spun. Finally, Figure 2(d) shows the fibers obtained from the 20% w/w PVA 2 solution; however, fewer fibers with much more dispersed diameters were detected. Considering the sequence, from this point on, only the nanofibers blow-spun from the PVA 2 18% w/w solution were tested, being mentioned as "PVA nanofibers".

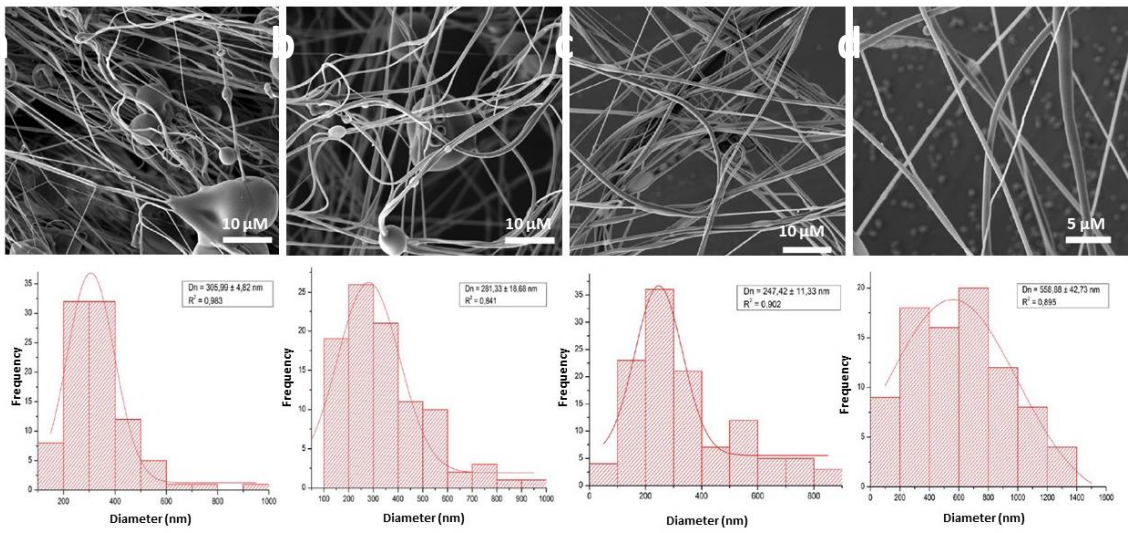
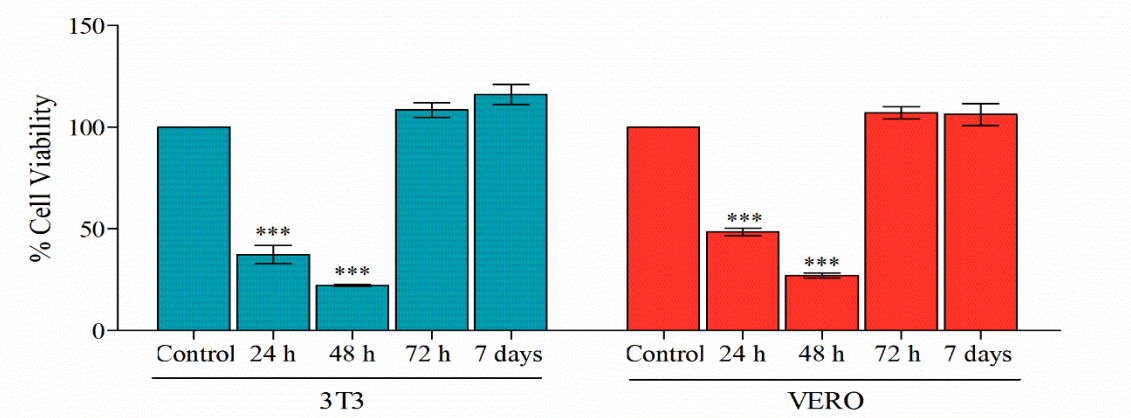


Figure 2: FEG-SEM images (and the respective histograms, below) of SBS fibers produced by PVA 2 solutions at (a) 15% w/w, (b) 16% w/w, (c) 18% w/w, and (d) 20% w/w.

3.4. Cell tests

In total, 3 types of cells (two cell lines and a primary culture cell tissue from rat spinal cord) were submitted to different methods to study their interaction with PVA nanofibers in elution method, as described before, having the follow cell lines: VERO and 3T3 – standardized for cytotoxicity materials tests (33) and resident spinal cord cells – final host cell population intended for nanofibers therapeutic application. The MTT assay was conducted to investigate the cell performance when exposed to PVA nanofibers eluted, with VERO and NIH 3T3 cell lines, in comparison to control (pure medium, FBS 10 %) same cell line seeded, with time exposition cut-off periods: 24 hours, 48 hours, 72 hours and 7 days. We found that after one day of incubation, both cell lines tested showed a significant reduction of viability ($p < 0.01$) which was exacerbated at 48 hours ($p < 0.01$), for VERO and 3T3. Conversely, the cell viability increased with additional incubation time, as at 72 hours and 7 days ($p > 0.01$), overcoming the control results (> 100%, standard for control) – with a small increasing trend for 3T3 (Graph 1).



Graph 1: Effect of incubation (cell viability) with PVA nanofibers elution after 24, 48, 72 hours and 7 days. Each column represents the mean ± SEM. (***$p < 0.01$ vs. control).

To reinforce the biological compatibility, a proliferative cell profile by the mitotic index was also determined. As shown before, a clear reduction in the mitotic index is observed 24 and 48 hours after exposure, translating an initially negative effect of polymer nanofibers over cell proliferation (48h, 3T3 and VERO, $p < 0.05$ vs control). However, the morphological profile of these cells might indicate a downregulation on cell-cycle progression (stopped at G0/G1 phases), since quickly after 72 hours post-exposure the frequency of mitotic cells seems to be initially restored ($p > 0.05$ vs control),

keeping the same trend for both cell lines after 7 days after exposure – greater than control for VERO cells – Fig. 3 – with representative images of mitotic events are shown for 3T3 , considering only late anaphase events, with the appearance of two discs migrating toward opposite poles of the cell, more representative from 72 h on, when a recovery effect on cell rate could be seen.

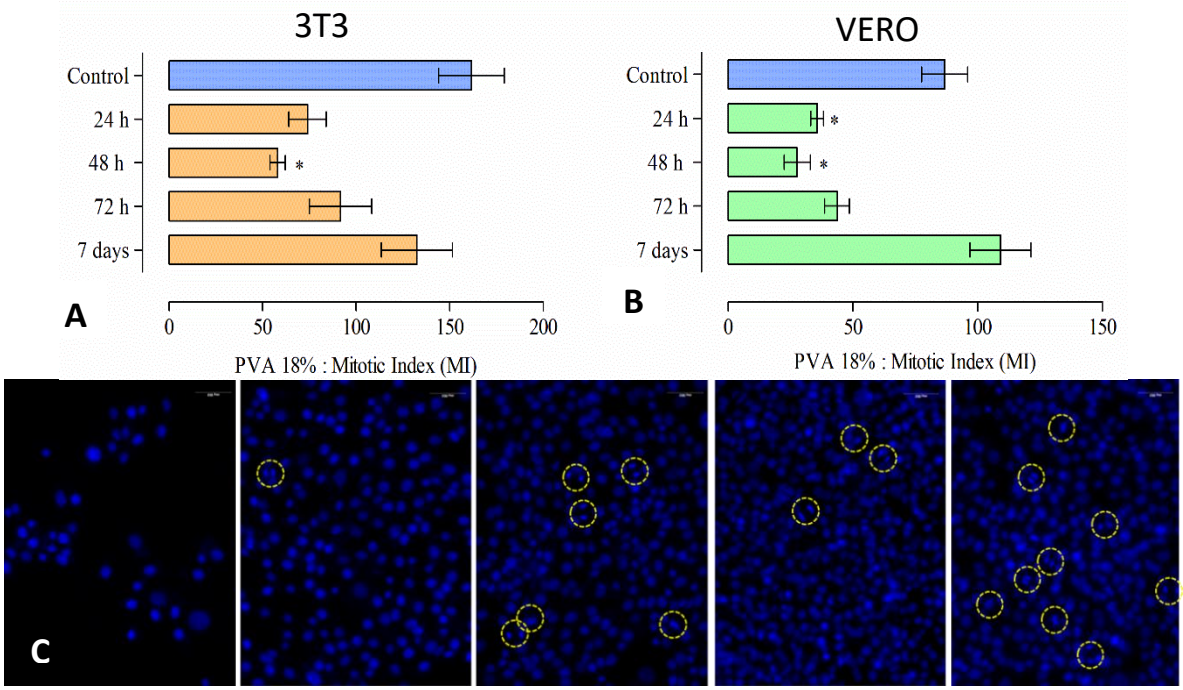


Figure 3: A and B -Mitotic index graphs after PVA nanofibers elution exposition at 24, 48, 72 hours and 7 days. Each column represents the mean ± SEM. (*p < 0.05 vs. control). C (line) - Representative images of VERO cells showing mitotic figures (yellow circles marking)

DAPI staining is also a tool that provides nuclear morphological features (area, eccentricity, and solidity) and might be related to several mechanisms that affect cell survival processes (33). The cell lines – 3T3 and VERO – and the resident spinal cord cells from medullary rat tissue – had their measurements of intensity dynamics of the nucleus by DAPI, showing the same trend between all of them: clear signs of apoptosis with disintegrated nuclei, as represented examples at Figure 4.

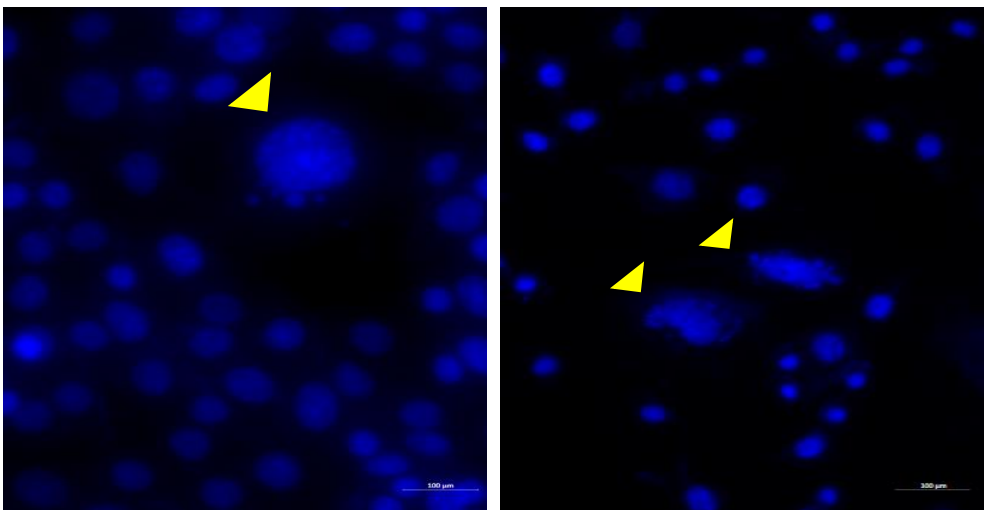
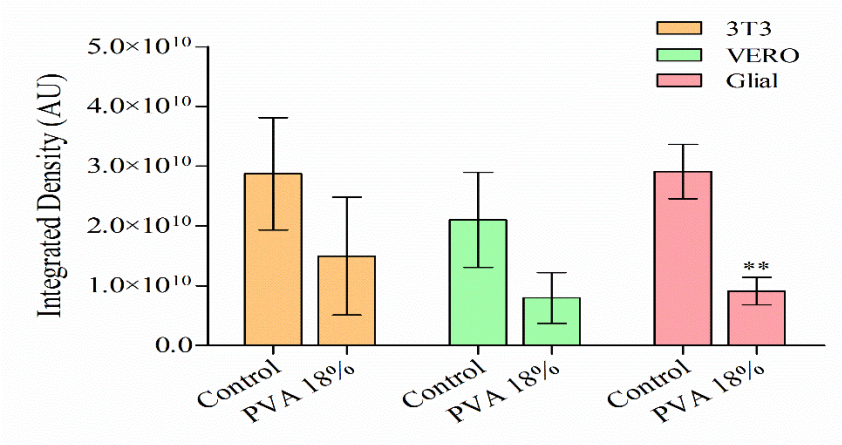


Figure 4: DAPI images of VERO cells after 72 hours exposure to PVA nanofibers elution; yellow markers point out to clear apoptotic nuclei.

Considering a non-favorable condition for cell proliferation, DAPI analysis were conducted with incubation with PVA nanofibers eluted until 72 hours, showing a clear decrease in their integrity

density (AU) rates for all cells, more pronounced in the resident spinal cord cells ($p < 0.05$ vs Control) – Graph 2.



Graph 2: DAPI Integrated density (AU) rates after 72 hours PVA nanofibers exposure.

The viability of VERO and resident spinal cord cells after elution exposition was also assessed by LIVE/DEAD Cell Viability Assay. For both cell types, comparing to control (without PVA nanofibers elution in media culture), up to 48 hours, a clear increase of dead cell percentage could be seen, meaning an "inhibition process" triggered by PVA exposition – with cell population kept alive. From 72h time point onwards, on the other hand, an inversion of that is seen, with more live cells than dead cells, meaning the existence of a favorable cell growth conditions. At the final point of analysis, 7 days, just a few of dead cells can be seen on images, having the same pattern as the initial control cell seeded (Fig. 5).

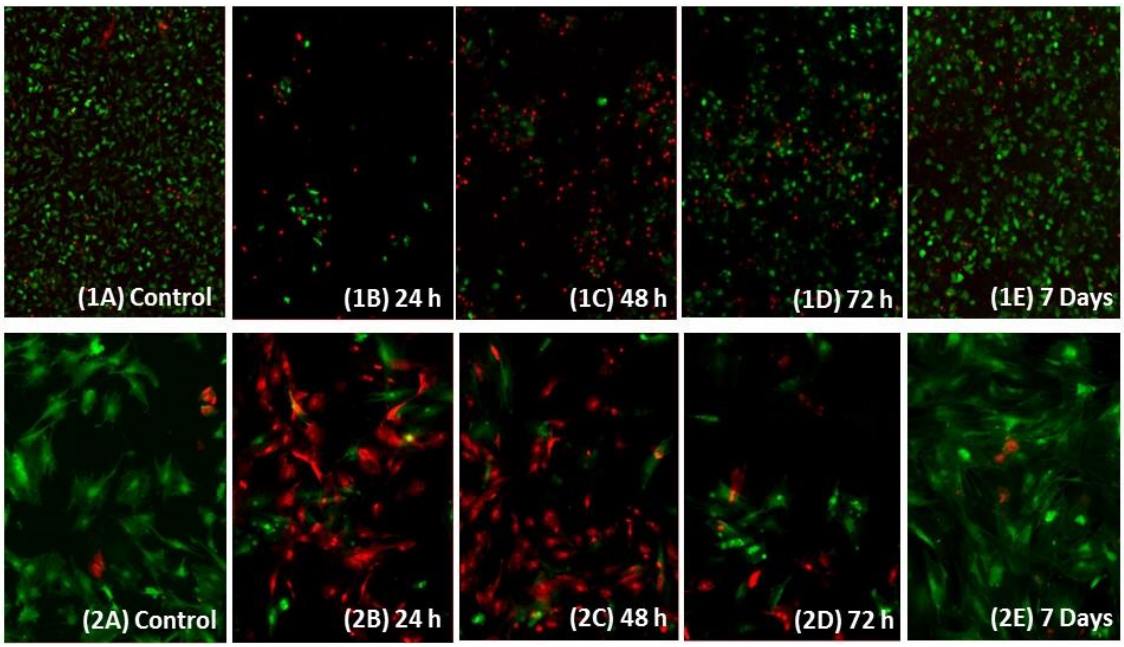


Figure 5: LIVE/DEAD cell viability assay; 1 (superior line sequence): VERO cells – A: control, without PVA nanofibers elute exposition, live (green) cells in major; B: 24h PVA nanofibers exposition, significant decrease of cell marked, possibility of quiescence phenomenon; C: 48h PVA nanofibers exposition, small increasing of dead cells (red); D: 72h PVA nanofibers exposition, a significant overlap of live cells (green); E: 7 days PVA nanofibers exposition, live (green cells) in clear majority. 2 (inferior line sequence): resident spinal cord cells – A to E: exactly same trend as VERO cells – first 48h showing the increment of dead (red) cells and then the overlap of live (green) cells (magnification; 1A-E: 40x; 2A-E:400x).

3.5. *Ex vivo* model

Considering the already described *ex vivo* models for spinal cord injury and therapeutic tests, an illustrative *ex vivo* model to elucidate the interaction of PVA nanofibers with animal medullary tissue was executed. Basically, a simple rat was euthanized and had its fresh spinal cord extracted, kept under controlled humidity conditions by a short interval, being transported and adapted as the final SBS apparatus system collector. Applying the already described SBS parameters and PVA 2 18% (w/w) solution, the nanofibers were directly spun to spinal cord during a fixed interval – 30 seconds – and studied directly afterwards in terms of polymeric nanofibers adhesiveness to medullary tissue and estimated degradation time interval, by surgical microscope images.

The PVA nanofibers showed high stickiness to rat spinal cord and adhered instantly when contact between them occurred. The fibers produced by fixed time interval proved able to cover all the spinal cord area exposed (uncovered), meaning a high nanofiber rate production by SBS system. In terms of degradation, considering the polymeric solution prepared with MilliQ as solvent, by sequential microscopic observation, after 24 hours, all the fibers were degraded, showing a high degradation rate of nanofibers and no inflammatory signs on tissue until 48 hours analysis (Fig. 6).

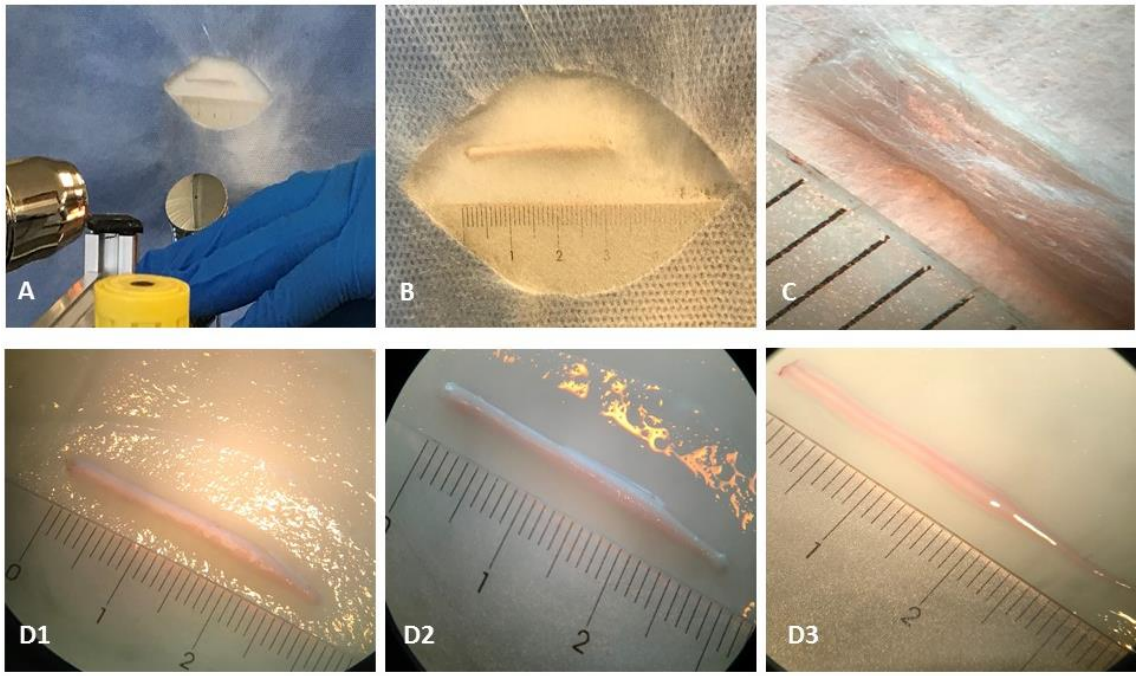


Figure 6: A – SBS apparatus producing PVA nanofibers having spinal cord as collector; B – rat spinal cord covered by SBS PVA nanofibers; C – dry partial rat spinal cord sample showing instantly nanofiber adhesion; D – surgical microscopic rat spinal cord *ex vivo* model images of time points (kept inside humidity chamber), 1: 30 minutes, 2: 24 hours, 3: 48 hours.

4. DISCUSSION

Currently, spinal cord injury is still considered a big challenge in terms of therapeutics, since there is a lack of effective results expressed by clinical improvement. In general, the concept of “time is spine” is applied by surgeons (34), based on the need for secondary lesion control as fast as possible, achieved by decompression and stabilization. According to regenerative medicine principles, the application of polymers in the context of SCI is considered highly promising, with the real possibility of acting directly on the lesion site, cooling down the secondary lesion – directly by polymer properties action, transporting active agents as cells or neuro growth factors, filling the cavitation defects (35). Still, reproducing the complex structural architecture of the extracellular matrix (2), as showed before by different strategies in literature (1).

Since the polymer nanofibers can mimic the local CNS architecture, promoting or stimulating

the local regenerative process in a secondary stage, the SBS technique appears as a new translational tool for this context. Described by Medeiros et al (13), showing many advantages over electrospinning, this nanofiber production technique has already been tested in different organs and systems, including in a "ready to use" surgical device (36). Showing a less complex apparatus than other techniques, the adjustment of all the parameters is critical for the success of SBS requiring extensive preliminary tests for standardization – working distance, nozzle diameter, gas pressure, temperature, and solution – as shown in this project. Taking the working distance, different from Zuidema et al (6) and Abdal-Hay et al (12), which showed 20 cm as ideal, and at the opposite, from Afanasiev et al (37) that used 40 cm, our 30 cm is in the between, still feasible for a potential translation to surgical context application. Different from most of literature (9, 19, 38), our ideal pressure for SBS nanofiber fabrication is higher, possibly due to solution characteristics, still close to others (10) and in the range of a common gas-powered surgical device – as a drill, for example.

Considering that the diameter of nanofibers has an important role in the arrangement and specific biological behavior of cells (39) and that with a smaller, more uniform and as good entanglement (creating the "pore spaces") as possible, a better effect on spinal cord repair has been seen (1, 40), this project chose de range 200-400 nm fibers diameter for a good behavior of CNS resident cells considering a translational future effect. In accordance with Santos et al (19), to achieve that, a heat source was required, differing from many authors who obtained 350 – 1000 nm fibers diameters by SBS technique (6, 41). Another critical point about SBS technique is regarding the solvent used for polymeric solution composition – most authors apply organic solvents, as chloroform (6, 9, 14, 37, 38), acetone (9, 14, 28) and others, based on SBS principle, that solvent evaporates totally during the working distance. On the contrary, considering a really sensitive tissue as medullary cord in a translational context, this project chose distilled water as solvent, in accordance with Santos (19) – adding more challenges to obtain complete solvent evaporation and good fibers diameters, but keeping the system totally safe for a possible future application on live tissue. Following the biological requirements to treat SCI by inhibiting apoptosis or necrosis and positively altering the local neurochemistry – reducing glial scar formation at the end stage (42) – the PVA already showed good results on this way applied by different techniques (43). The choice of this synthetic polymer was based on good results obtained, considering biocompatibility and degradation rates under physiologic conditions (44, 45). In accordance to Hiraizumi et al (27) who applied a PVA hydrogel membrane on spinal cord tissue decreasing the local inflammatory reaction and scar tissue formation, the results presented by this project considering cell tests, showed an initial clear decrease on cell metabolism and dynamics, resulting on lower cell viability rate, mitotic index, and percentage of live cells until 48h exposition, cooling down the environment. On the other hand, after this time point, all cell types tested, including rat resident spinal cord cells, showed an increase in their metabolism, expressing a possible good microenvironment for regeneration. Again, considering translational needs for biomaterial application in the SCI context, this can represent a first control to the secondary lesion extension and a sequential creation of local condition for axonal regeneration (1).

Different from most synthetic polymers (46), and the results shown by Hiraizumi (27) and Comolli (43) with PVA, the nanofibers generated by SBS in this project showed a high degradation rate and good adhesion to local tissue, becoming adhered without pressure just by contact, as showed by *ex vivo* model, not microscopically identified after 24 hours – probably because of the nanofiber diameter and watery solution. These points are interesting, since the potential translation allows a non-pressure active biomaterial deposition directly over a previous damaged and sensitivity tissue. Another point of consideration can be extracted from Yang et al (47), who tested 3T3 cells on different PVA nanofibers, showing cell-cell communication just after 72 hours after cell seeding experiments, with ECM productions on day 7 – possible similar mechanisms for our positive results from third day on, also with cell number increase.

This project has limitations, mainly based on the influence of external ambient characteristics over the result of the SBS apparatus. Moreover, the mechanisms of polymer interaction with cells were not evaluated, leading to new questions and possibilities of future studies and interventions. Additionally, for the first in the literature, this project presents the potential advantages of SBS

technique fabricating PVA nanofibers aimed translationally at SCI application – considering all the sterile conditions required and feasible costs involved. Further investigations are needed and encouraged for optimization (association with cell spray, active molecules, and conductive materials, for example) until a final translation to the spine surgeon as an effective therapeutic tool, providing clinical results to the patients.

5. CONCLUSION

The solution blow spinning technique proved to be an achievable technique to produce PVA polymer nanofibers with standardized characteristics, considering the application of a biomaterial solution in distilled water as solvent. Cell tests and *ex vivo* model demonstrated a feasible interaction with polymer PVA nanofibers, resulting in determination of a prototype with translational potential to spinal cord injury therapeutic context and constituting a new strategy for subsequent investigations.

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Acta Biomaterialia

POLYMER NANOFIBERS PRODUCED BY SOLUTION BLOW SPINNING METHOD – AN INNOVATIVE EXPERIMENTAL PROTOTYPE TOOL TO SPINAL CORD INJURY

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full length article
Section/Category:	Opt in to First Look
Keywords:	spinal cord injuries; polymers; biodegradable nanofibers; tissue engineering; in vitro studies.
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Abstract:	Spinal cord injury (SCI) has drastic effects, having limited results by actual available therapeutic. Focusing on the need of control of secondary lesion, the regenerative medicine through tissue engineering brings promising options. Applying polymers in nanofibers can be a rational approach, once acting directly on lesion site. There are many techniques for that, being the solution blow spinning (SBS) easier and with possibilities of effective translation. This project aims to investigate the potential of PVA nanofibers in aqueous solution produced by SBS as a translational therapeutic prototype for SCI. Technical parameters were standardized, and cell cytotoxicity tests were performed by, MTT assay, DAPI, Live/Dead cells. Based on an ex vivo model, the adhesiveness and degradation rate were estimated. Nanofibers with adequate FEG-SEM image characteristics were produced, showing a first negative impact with cell contact, allowing recovery from medium-long timepoints exposition (72 hours – 7 days); interesting adhesiveness and degradation rate were showed by ex vivo animal model. The SBS technique proved to produce PVA nanofibers having water as solvent, with interesting positive results, being considered as a prototype with translational potation to SCI therapeutic context.

UNIVERSIDADE DE CAXIAS DO SUL

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CEUA-UCS

Caxias do Sul, 29 de maio de 2020

Of.CEUA 11/2020

A Comissão de Ética no Uso de Animais vem por meio deste autorizar a alteração proposta no Projeto no. 014/2018, intitulado "EFEITOS DA NICOTINA NA LESÃO MEDULAR TRAUMÁTICA ASSOCIADA AO TRATAMENTO COM OXIGENOTERAPIA HIPERBÁRICA", do Pesquisador Prof. Asdrubal Falavigna, levando em consideração os problemas enfrentados durante o fechamento dos laboratórios e a interrupção das atividades de pesquisa ocorridos nos meses de março e abril de 2020 devido à pandemia da COVID-19. Fica aprovada a complementação de 13 animais no número amostral, conforme tabela abaixo.

FINALIDADE	( ) ENSINO (X) PESQUISA CIENTÍFICA
Vigência da autorização	12/2020
Espécie / linhagem / raça	Ratos Wistar
No. de animais	13
Peso / idade	200g / 6-8 semanas
Sexo	Machos
Origem	CREAL-UFRGS

Atenciosamente,

Prof. Dr. Matheus Parmegiani Jahn

Coordenador CEUA/UCS

Caxias do Sul, 18 de Novembro de 2020.

Ao Prof. Dr. Matheus Parmegiani Jahn
Coordenador da CEUA/UCS

Prezado Coordenador,

Solicitamos a sua atenção acerca do projeto de pesquisa intitulado "**Efeitos da Nicotina na Lesão Medular Traumática Associada ao Tratamento com Oxigenoterapia Hiperbárica**", o qual foi aprovado pela CEUA/UCS em 30 de agosto de 2018 (Of CEUA 015/2018; número 014/2018).

O projeto supracitado prevê o uso de ratos *Wistar* de 6 semanas de idade (N = 9) utilizando o modelo *ex-vivo* para a realização do traumatismo raquimedular TRM, o qual utiliza fragmentos de medula extraído dos ratos, imediatamente após a eutanásia por overdose com isoflurano.

Durante a realização do modelo, ao coletar o sobrenadante do meio de cultivo onde o tecido medular fica imerso, se observou a possibilidade de isolar células gliais para o estabelecimento de cultura celular. Neste sentido, observamos a possibilidade de utilizar essas células isoladas para cultivo e armazenamento em ultrafreezer (-80°C), prevendo a utilização destas em outros projetos envolvendo TRM, tema do grupo de pesquisa. É importante salientar que este sobrenadante é descartado durante as trocas de meio de cultivo para a manutenção do modelo *ex-vivo* por 14 dias.

Frente a esta possibilidade, gostaríamos da apreciação e aprovação da Comissão de Ética para Uso de Animais da Universidade, a fim de utilizar ao máximo o tecido extraído destes animais em experimentação, prevendo a preservação da utilização de mais animais em projetos futuros que necessitam de células residentes da medula espinhal.

Cordialmente,

Drª Natália Fontana Nicoletti Roxo
Responsável Técnica do Laboratório de Terapia Celular

Dr. Asdrubal Falavigna

4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A partir dos resultados obtidos e apresentados por esse projeto, em suma, traz-se a possibilidade efetiva de aplicação da técnica SBS para fabricação de fibras poliméricas, tendo-se água como solvente, direcionando-se os parâmetros para possibilidade de translação em contexto de aplicabilidade cirúrgico-terapêutica em arsenal para a lesão medular.

A elevada adesividade verificada pelo teste *ex vivo* ilustrativo, bem como taxa de degradação em ambiente nativo destacam duas vantagens quanto ao polímero escolhido, PVA, bem como formação e disposição do material composto em estrutura por nanofibras entremeadas – a princípio, sem exercer pressão significativa sobre o anteparo (coletor), ou destino final de aplicação por SBS.

Em relação à citotoxicidade do PVA em nanofibras, por meio de testes padronizados em literatura, aplicando-se modelos de linhagens celulares pré-estabelecidos, bem como ampliando para verificação frente às células e ambiente nativo tecidual medular, destaca-se o impacto negativo inicial pela interação entre as fases (células x nanofibras de PVA), em oposição à completa recuperação e, dependendo do tipo celular e teste celular específico, sobreposição ao controle (cultura sem exposição ao biomaterial em nanofibras), inferindo-se uma possibilidade de ação de propriedade inerente ao próprio polímero – com registros em literatura de causar minimização de reações de cascata inflamatória e cicatricial não-funcional – a qual, em correlação ao TRM, poderia configurar elevado nível de aplicabilidade e interesse por, teoricamente, ser capaz de interromper ou minimizar o mecanismo deletério da conhecida lesão secundária, imediatamente após sua aplicação, ainda possibilitando, em segundo momento, adaptabilidade natural do ambiente para atuação de mecanismos reparadores e de manutenção celular nativa – correspondendo à aplicação dos princípios da engenharia de tecidos e medicina regenerativa associados.

Os mecanismos bioquímicos e reações envolvidas quanto aos resultados apresentados não estão incluídos nos objetivos determinados para desenvolvimento desse projeto, porém motivam a sequência de novos estudos quanto ao tema e linha e pesquisa da Instituição, permitindo otimizações subsequentes.

A partir da demonstração da técnica SBS como factível e viável para fabricação de nanofibras em modelo prototipado aplicável aos passos futuros de translação potencial, considerando-se a possibilidade de esterilização de todo o aparato, uso de

fontes de gases e pressão disponíveis para aparelhos rotineiros em ambiente cirúrgico, bem como distância de trabalho passível de execução em contexto real, considera-se que possa ser iniciado nova linha de pensamento para desenvolvimento de técnica e eventualmente produto final em ciências da saúde, respeitando-se as etapas científicas e de regulação ética. Destaca-se também, que, recentemente, tem-se publicações de dispositivos em comercialização em outros países que aplicam a mesma técnica, porém, em diferentes contextos finais, polímeros, soluções, objetivos terapêuticos e características das fibras, tendo-se o enfoque positivo na real possibilidade de desenvolvimento de aparato de aplicação em ambiente cirúrgico humano.

Considerando passos futuros propostos, a partir dos resultados apresentados para esse projeto, destacam-se:

- Adição de moléculas bioativas em solução polimérica com fins de diminuir o impacto negativo inicial de interação entre as nanofibras e as células locais (a exemplo de proteínas – fatores neurotróficos);

- Associação de segundo sistema de sopro em solução, destinado à aplicação conjunta de células em meio de cultura, pré-diferenciadas para fenótipo do sistema nervoso central e/ou indiferenciadas, sob baixas pressões de ar (mantendo-se o sistema SBS, porém sem necessidade de fabricação de fibra), tendo-se como alvo comum a mesma área coletora no anteparo que as nanofibras, possibilitando que as células aplicadas permaneçam incluídas na estrutura polimérica entremeada que simula o ambiente da matriz extracelular nativa, com possibilidade de manutenção da viabilidade celular dessas e interação como mecanismo adicional de regeneração externa, aumentando as possibilidades de melhorias funcionais finais;

- Combinação do polímero com outras estruturas orgânicas com potencial funcional final, considerando o SNC; traz-se como exemplo inicial o grafeno – nanomaterial composto por carbono, sabidamente com elevada biocompatibilidade e condutor de eletricidade, o que, em tese, poderia estimular a diferenciação de células primordiais em células condutoras de eletricidade, compondo nanofibras funcionais e aumentando as possibilidades de resultados finais positivos quanto à resposta de recuperação motora e sensitiva;

- Delineamento e execução de modelo animal de trauma raquimedular tratado comparativamente e avaliado, funcionalmente e prospectivamente, pela técnica de nanofibras depositadas em medula fabricadas pela técnica SBS,

Por fim, considerando o exposto até o momento, destaca-se a suma importância e ocorrência para esse estudo de colaboração multidisciplinar, considerando os diferentes campos e áreas de conhecimento envolvidos em desenvolvimento de tecnologia de potencial translacional cirúrgico humano.