Available online at www.sciencedirect.com

ScienceDirect

Ciência & Tecnologia dos MATERIAIS

http://ees.elsevier.com/ctmat

Ciência & Tecnologia dos Materiais 29 (2017) e135–e139

Special Issue "Materiais 2015"

Neuro-muscular regeneration using scaffolds with mesenchymal stem cells (MSCs) isolated from human umbilical cord Wharton's jelly

A.R. Caseiro[a,b,c], T. Pereira[a,b], J. Ribeiro[a,b,d], J.D. Santos[c], I. Amorim[e,f], A.L. Luís[a,b,d], A.C. Maurício[a,b,*]

[a]*Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP), Porto, Portugal*
[b]*Centro de Estudos de Ciência Animal (CECA), Instituto de Ciências, Tecnologias e Agroambiente (ICETA), Porto, Portugal*
[c]*REQUIMTE/LAQV – U. Porto – Porto/Portugal, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal*
[d]*UPVET, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP), Porto, Portugal*
[e]*Departamento de Patologia e de Imunologia Molecular, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto (UP), Porto, Portugal*
[f]*Instituto Português de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP), Porto, Portugal*

Abstract

Peripheral nerve regeneration following severe events is still a challenging topic in the regenerative medicine field, especially when nerve tissue is lost and direct suturing is not feasible. Given the limited success observed in currently available techniques, researchers have been putting efforts towards the development and optimization of these techniques, aiming at the best recovery chances for affected patients. The present work explores the combination of two methods of synthetic biomaterial tubes functionalization: the effect of electroconductive biomaterials and its association to an active cellular system. A tube-guide comprised of polyvinyl alcohol (PVA) loaded with COOH-functionalized multiwall carbon nanotubes (CNTs) was produced and studied alone or in combination with a cellular system of mesenchymal stem cells (MSCs) isolated from the umbilical cord Wharton's jelly (WJ). Tube-guides were assessed for *in vitro* cytocompatibility to the WJ MSCs and tested for *in vivo* performance in a neurotmesis rodent model. Animals were assigned to either PVA-CNTs, PVA-CNTs-MSCs, graft or end-to-end reconstruction groups and assessed after 20 weeks of regeneration. Structural analysis revealed overall more evident recovery in the PVA-CNTs-MSCs group, with significant differences to the cell-free group and similar to End-to-End repaired and Grafted groups. Surprisingly, PVA-CNTs-MSCs did no benefit neurogenic muscle atrophy recovery. Overall, the electrofucntionalized tube-guides post an interesting option for nerve reconstruction alone or in combination to MSCs cellular systems.

Keywords: Mesenchymal stem cells; nerve regeneration; neurotmesis; biomaterials; histomorphometry, Wharton's jelly.

1. Introduction

Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery, as functional recovery is rarely satisfactory in these patients [1-4]. Direct repair should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a gap need graft implantation [1,3]. Entubulation offers advantages, including the potential to manipulate the regeneration environment within the tube-guide, adding cellular systems and/or growth factors inside its lumen [1-4]. Keeping in mind the

* Corresponding author.
E-mail address: ana.colette@hotmail.com (A.C. Maurício)

http://dx.doi.org/10.1016/j.ctmat.2016.04.003

electrophysiology nature of the neuromuscular tissue, and the knowledge that electrical events influence its homeostasis and regeneration, electro-functionalised biomaterials arise as interesting options to explore. It has been demonstrated that low frequency stimulation leads to improved electrophysiological recovery and myelinated fibres content in reconstructed nerves [5,6]. The inclusion of the stimulatory effect as an attribute to the implantable nerve conduit is the rational for the development of electroconductive biomaterials as explored herein. Another option for functionalizing neuro-reconstruction biomaterials is the association of a cellular component. As recently revised in [7], several studies detail the effects of the association of MSCs' systems on nerve injury models. Majorly, these seem to impact on the inflammatory and degenerative events, as well as on diverse regenerative parameters (myelin sheaths thickness; fibre numbers and organization, re-vascularization and fibrosis reduction) [3,8,9]. The MSCs therapeutic effect does not simply reside on their capacity to replace original cells of damaged tissues, but also by secreting growth factors and cytokines that modify the microenvironment inducing the activity of endogenous regenerative cells within the injured tissue [3,10-12], and by modulating the inflammatory and immune responses [2-4,13-15]. Therefore, the use of cellular systems is a rational approach for delivering growth-promoting factors and cytokines at the nerve lesion site [2-4,13-15]. Additionally, the culture media enriched in growth factors produced by MSCs in expansion may become an important alternative to the MSCs application [14,15].

Currently, the bone marrow (BM) represents the main source of MSCs [7]. However the number of BM-MSCs significantly decreases with age and the HLA compatible donors are very difficult to find. The MSCs obtained from the umbilical cord tissue (UCT) are a promising alternative: i) the number of cells per volume is higher, ii) because of the low expression of HLA-ABC antigens and the absence of HLA-DR expression, a complete or high HLA profile match for allogenic use is not necessary, which permits to greatly enlarge the number of available donors, and the use in xenografts, iii) are easier to obtain, cryopreserve, and the collection is ethically approved by national and international laws, iv) the number of high quality samples cryopreserved is increasing in public and private cord blood banks in Portugal and Europe [4]. The goal of the present work was to evaluate the therapeutic effect tube-guides with electrical conductivity for nerve regeneration associated to WJ MSCs on neuro-muscular regeneration after neurotmesis injury using the rat sciatic nerve model. For such, a matrix of polyvinyl alcohol (PVA) was produced and loaded with electrical conductive COOH-functionalized multiwall carbon nanotubes (CNTs). MSCs from the WJ have been reported in experimental trials as cell-based therapies, including pathologies of peripheral and central nervous system [7].

2. Materials and Methods

The isolation of WJ MSCs, their expansion and characterization has already been described by our research group for fresh and cryopreserved UC tissue, [2-4]. We have also previously reported the differentiation of the MSCs into neuroglial-cells and studies their secretome and metabolomics profiles [2-4]. In the present study, human MSCs (MSCs) isolated from the Wharton jelly (WJ) of umbilical cord (UC) were cultured and maintained in Mesenchymal Stem Cell Medium, PromoCell (C-28010), in a humidified atmosphere with 5% CO_2 at 37ºC. MSCs were expanded until significant numbers were attained (Fig. 1 b.)). Cells were harvested with 0.25% trypsin with EDTA (GIBCO), and a cell suspension was prepared in 1 mL syringes, containing MSCs at a concentration of 10^6/µL for posterior intra-operatively nerve injection. Biomaterials used for tube-guide development were based on a matrix of PVA loaded with electrical conductive CNTs. Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10-98) loaded with COOH-functionalized multiwall carbon nanotubes CNTs (Nanothinx, NTX5, MWCNTs 97% -COOH) (PVA-CNTs tube-guides) were prepared using a casting technique to a silicone mould. The 15% (wt./vol.%) aqueous solution of PVA was mixed with 0.05% of COOH-functionalized CNTs and entered a freezing/thawing process of three cycles of freezer (-30ºC)/incubator (25ºC). An annealing treatment was initiated at 25ºC for 14h followed by an increasing stage at a rate of 0.1ºC/min until 80ºC, and finalized for 20h at 80ºC. The tube-guides were sterilized by gamma-radiation. Before surgical implantation, tube-guides were hydrated in a sterile saline solution for 2h (Fig. 1 a)). The tube-guides were associated to MSCs isolated from the WJ (Fig. 1 b)) and tested *in vitro* for cytocompatibility prior to *in vivo* application. For this step, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in Fura-2-loaded MSCs cells by using dual wavelength spectrofluorometry as previously described [16].

Results obtained from epifluorescence technique are referred to measurements from MSCs which correspond to ($[Ca^{2+}]_i$) from cells that did not begin the apoptosis process (data not shown) alone, sub-cultured over PVA-CNTs discs of 10 mm diameter.

For the *in vivo* application, adult male Sasco Sprague Dawley rats weighing approximately 300 g were used.

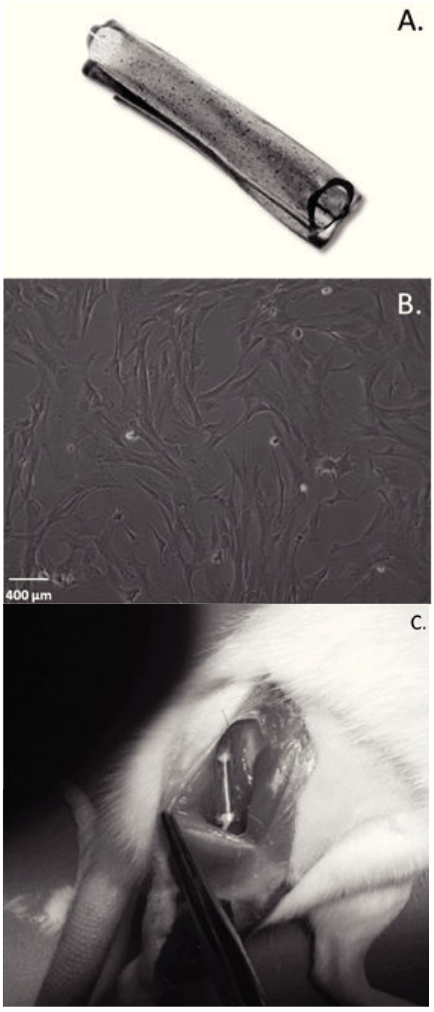

Fig. 1. a) Tube-guide composed of PVA loaded with electrical conductive COOH-functionalized CNTs, b) Human UC-WJ MSCs at passage 5 in adherent culture (Magnification 100x), c) Surgically implanted tube-guide, bridging the 10 mm gap between the sectioned nerve stumps, as applied in groups PVA-CNTs and PVA-CNTs-MSCs.

All the procedures involving animal handling and experimentation were assured in conformity with applicable national and international guidelines and legislation (Directive 2010/63/EU of the European Parliament and with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC)). Animals were routinely prepared for surgery and anesthetized (ketamine 9 mg/100 g; xylazine 1.25 mg/100 g, g body weight, intraperitoneal). The right sciatic nerve was exposed and mobilized from the surrounding tissue. The nerve was transected above the terminal nerve ramification. The nerve stumps were inserted 3 mm into the tube-guides, maintaining a gap of 10 mm, and fixated using 7/0 monofilament polypropylene epineural sutures (PVA-CNTs group). For the group including MSCs (PVA-CNTs-MSCs group), the tube-guide was filled with 100 µm of MSCs at a concentration of 10^6 cells/µL in culture medium, and 100 µm of MSCs at the same concentration were infiltrated in both nerve stumps inserted in the tube-guide (Fig. 1 c.)). Two additional groups were included with graft (Graft group) repair and end-to-end suture (End-to-End group), by suturing an autologous 180º-inverted graft between the nerve stumps, or directly suturing the sectioned stumps, respectively. Nerve regeneration was observed after 20 weeks of implantation. At the endpoint, histomorphometric analysis was performed to assess for the nerve morphologic recovery. Prior to euthanasia, nerves were collected under deep anesthesia, and fixated in 2.5% purified glutaraldehyde and 0.5% saccharose in 0.1M Sorensen phosphate buffer for 6-8h and resin embedded following Glauerts' procedure. Series of about 50 semi-thin transverse sections (2-µm thick) were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) starting from the distal end of the specimen and stained by Toluidine blue. Stereology was carried out on one section, randomly selected each of these series, using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

The *tibialis anterior* (TA) muscles were also collected and fixed in 10% buffered formalin, routinely processed, dehydrated and embedded in paraffin wax. Consecutive 3 µm transverse sections from the mid-belly of each muscle were cut, stained with haematoxylin and eosin (H&E) and used for morphometry evaluation and determination of the degree of atrophy.

For the morphometric analysis, an unbiased sampling procedure was applied and the following measures were calculated: area, perimeter, “Feret’s angle” and “minimal Feret’s diameter” (which is the minimum distance of parallel tangents at opposing borders of the muscle fibre). These parameters were evaluated from the cross sections using the ImageJ© software (NIH) which allowed to apply this set of individual fibre measurements. A minimum of 1000 skeletal muscle fibres were measured from each group. This assessment was performed by two independent operators. Each one of the operators measured, blindly and randomly, an average of 50 fibres in each section. Images were acquired using a Nikon® microscope connected to a Nikon® digital camera DXM1200, at

low magnification (100x) under the same conditions that were used to acquire a reference ruler.

After complete necropsy, histopathology of lung, liver, kidneys, and regional lymph nodes was also performed to ensure the biocompatibility of these biomaterials. Specifically, we investigated any signs of systemic toxicity or accumulation of CNTs in these organs after biodegradation. The organs were fixed in 10% buffered formalin and processed for routine histology with H&E stain.

Massive microscopic carbon deposits might be observed in the lung or as small punctuated accumulations inside Kupfer cells (liver) and in the intermediate zone of the spleen, when intravenously administered. Von Kossa staining (to evidence phosphates and carbonates) and Masson-Fontana (that distinguishes carbon deposits from melanin) were also performed in consecutive histologic sections, in order to ensure that these deposits were not present in the treated animals.

3. Results

WJ MSCs were expanded up to P5 prior to application (Fig. 1 b)), retaining their characteristic fibroblast-like spindled shape and chromosomal stability, as assessed by karyotyping studies (data not shown).

PVA-CNTs tube-guides were successfully produced and surgically implanted. Cytocompatibility measurements from epifluorescence technique on undiffentiated MSCs correspond to $[Ca^{2+}]_i$ from cells that did not begin the apoptosis process. The $[Ca^{2+}]_i$ was 46.2±3.5 nM (N=25) for MSCs cultured in the presence of PVA-CNTs discs after 7 days of culture. The cultured MSCs reached confluence and exhibited a normal star-like shape with a flat morphology in culture. Histomorphometric analysis of nerves recovered after 20 weeks depicted nerve fibre regeneration in all repaired nerves (Table 1). Microfasciculation events were more evident in the PVA-CNTs-MSCs repaired group. Also, MSCs association to the electroconductive tube-guides resulted in a significantly increased myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fibre diameter (d/D; g-ratio) when compared to PVA-CNTs group ($P < 0.05$). Comparing to End-to-End and Graft groups, PVA-CNTs-MSCs group did not significantly differ in three stereological parameters (M, M/d, and g-ratio). Regarding neurogenic muscle atrophy, it was observed a significant increase ($P < 0.05$) in mean fibre size between the PVA-CNTs and the Graft group, displaying 42% increase in terms of average fibre area and a 21% increase in term of the "minimal Feret's diameter". However, obtained values were still significantly lower ($P < 0.05$) than undamaged nerves/muscles. The PVA-CNTs-MSCs displayed macroscopically smaller muscles and decreased TA fibre. Areas of necrosis and delayed muscle regeneration were also observed. At macro and microscopic examination, no alterations were observed in the experimental animal's internal organs. Microscopic analysis confirmed the absence of inflammation, cell degeneration, necrosis and fibrosis. No signs of nanotubes, neither carbon deposits were detected in all collected organs (Fig. 2).

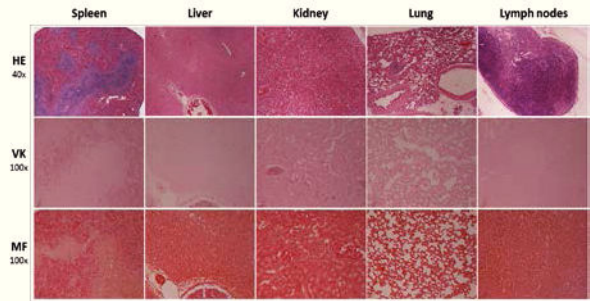

Fig. 2. Microscopic examination of the spleen, liver, kidney, lung, and regional lymph nodes of the PVA-CNTs and PVA-CNTs-MSCs groups did not reveal any abnormalities compatible with systemic toxicity or accumulation of biodegradation products of CNTs. Haematoxylin and eosin (HE)(Magnification 40x), Von Kossa staining (VK)(Magnification 100x), and Masson-Fontana (MF)(Magnification 100x).

Table 1. Stereological quantitative assessment.

Group		Density	Total number	Axon diameter (d)	Fibre diameter (D)	Myelin thickness (M)	M/d	D/d	d/D (g-ratio)	Area (mm^2)
End-to-end	ẍ	38,762	22,729	2.65	3.58	0.47	0.19	1.38	0.73	0.5990
(N=5)	σ	1,524	2,308	0.14	0.16	0.01	0.01	0.01	0.01	0,2082
Graft	ẍ	37,041	21,939	2.58	3.56	0.49	0.21	1.41	0.71	0.5936
(N=4)	σ	1,214	1,302	0.06	0.08	0.02	0.01	0.02	0.01	0.0694
PVA-CNTs	ẍ	36,261	16,049	2.98	3.71	0.37	0.14	1.28	0.79	0.4455
(N=7)	σ	4,267	1,172	0.25	0.27	0.01	0.01	0.03	0.01	0.1856
PVA-CNTs-MSCs	ẍ	43,373	25,731	2.44	3.34	0.45	0.21	1.41	0.72	0.5942
(N=7)	σ	3,881	3,386	0.20	0.21	0.06	0.04	0.08	0.01	0.1940

Stereological quantitative assessment of the regenerated nerves - density, total number, axon diameter (d), fibre diameter (D), myelin thickness (M), ratio myelin thickness and axon diameter (M/d), ratio fibre diameter and axon diameter (D/d), ratio axon diameter and fibre diameter (d/D, g-ratio) of regenerated sciatic nerve fibres at week-20 after neurotmesis. Values are presented as ẍ ± σ. N corresponds to the number of animals within each experimental group.

4. Discussion and Conclusions

Our observations support the hypothesis that the use of synthetic tube-guides for the severe peripheral nerve might come as a valid alternative to currently applied surgical technique, such as nerve grafting and end-to-end suturing, especially when tension free reconstruction is not achievable. Synthetic PVA-CNTs were able to support WJ MSCs adhesion and growth. Cytocompatibility results obtained from epifluorescence by measuring the $[Ca^{2+}]_i$ of the MSCs cultured on biomaterials confirmed the ability to support their adhesion and expansion. Besides the physical support for the MSCs maintenance at the lesion site, such electroactive materials are expected to exert modulatory activities on MSCs, as observed on bone marrow MSCs [17]. Here CNTs were demonstrated capable of modulating MSCs activity, enhancing the expression of neuronal markers and neurotrophic growth factors. The PVA-CNTs tube-guides proved safe to use in the rat model as no traces of adverse systemic reactions to these or their degradation products were observed and no trace of carbon deposits was identified after 20 weeks of implantation. Electrical events are known to affect nerve function and recovery [5,6] and this positive effects were confirmed by the satisfactory performance of the PVA-CNTs alone. Structural recovery of the section nerves was also supported by both the cell free and the cell loaded systems. MSCs association to the biomaterial system tended to display superior stereological indexes, closely matching those of the grafted and end-to-end treated nerves, opening ground to their use as valid reconstructive alternatives. PVA-CNTs-MSCs promoted increased thickness of the regenerating myelin sheath as well as increased myelin thickness/axon diameter and r axon diameter/fibre diameter ratios, suggesting increased stimulatory potential. Nevertheless, TA muscle atrophy examination presented unexpected results. Cell free PVA-CNTs displayed improved recovery, while the presence of WJ MSCs did not benefit skeletal muscle parameters. Indeed, MSCs seemed detrimental in terms of neurogenic atrophy recovery. These controversial results deserve further study and comprehension, since literature commonly reports otherwise, as both as differentiated and undifferentiated MSCs from different sources have been associated to positive effects in counteracting neurogenic atrophy [18,19]. In conclusion, electrofunctionalized tube-guide might rise interesting options for the surgical reconstruction of otherwise unrepairable damages. The addition of active cellular components further boost the potential of such biomaterials, acting as trophic agents for the intrinsic regenerative populations, such as the injury activated Schwann cells [7]. Further studies are necessary to address function recovery and skeletal muscle atrophy recovery.

Acknowledgements

This research was supported by Projects Pest-OE/AGR/UI0211/2011 (FCT) and “iBone Therapies: Terapias inovadoras para a regeneração óssea”, NORTE-01-0247-FEDER-003262 (COMPETE). AR Caseiro has a PhD grant SFRH/BD/101174/2014.

References

[1] G. Lundborg, J. Peripher, Nerv. Syst. 7(3) (2002) 139.
[2] J. Ribeiro *et al.*, International Review of Neurobiology, I.P.P.T. Stefano Geuna, B. Bruno (Ed.), Academic Press, 2013, pp. 79-120.
[3] A. Gärtner *et al.*, J. of Stem Cells & Regenerative Med. X(1) (2014).
[4] A.C. Maurício *et al.*, Biomaterials Applications for Nanomedicine, R. Pignatello (Ed.), InTech, 2011.
[5] M.-C. Lu *et al.*, Neurorehabilitation and Neural Repair 22(4) (2008) 367.
[6] C.-C. Yeh *et al.*, Neurorehabilitation and Neural Repair 24(8) (2010) 730.
[7] A.R. Caseiro *et al.*, Stem Cells Int. 16 (2016).
[8] H.C. Pan *et al.*, J. Clin. Neurosci. 14(11) (2007) 1089.
[9] J.R. Lavoie, M. Rosu-Myles, Biochimie 95(12) (2013) 2212.
[10]J.W. Han *et al.*, Cell Transplant. 25(2) (2016) 313.
[11] L.P. Cartarozzi *et al.*, Brain Res. Bull. 112 (2015) 14.
[12] A. Gärtner *et al.*, Differentiation 84(5) (2012) 355.
[13] M. Dominici *et al.*, Cytotherapy 8(4) (2006) 315.
[14] T. Pereira *et al.*, PLoS One 9(11) (2014) 113769.
[15] T. Pereira *et al.*, Stem Cells Int. (2014) 376918.
[16] J. Rodrigues *et al.*, Biomedical Mater. and Eng. 15(6) (2005) 455.
[17] J.H. Lee *et al.*, Acta Biomater. 10(10) (2014) 4425.
[18] D. Schaakxs *et al.*, Muscle Nerve 47(5) (2013) 691.
[19] Y.-Y. Hsueh *et al.*, Biomaterials 35(7) (2014) 2234.