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NOTE

Induction of neurite outgrowth in 3D hydrogel-based environments

Rita C Assunção-Silva[1,2,4], Cátia C Oliveira[1,2,4], Ofra Ziv-Polat[3], Eduardo D Gomes[1,2], Abraham Shor[3], Nuno Sousa[1,2], Nuno A Silva[1,2] and António J Salgado[1,2]

[1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4701-057 Braga, Portugal
[2] ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal
[3] NVR Research Ltd, Ness-Ziona, 74031, Israel
[4] R C Assunção-Silva and C C Oliveira contributed equally to this work.

E-mail: asalgado@ecsaude.uminho.pt

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Abstract

The ability of peripheral nervous system (PNS) axons to regenerate and re-innervate their targets after an injury has been widely recognized. However, despite the considerable advances made in microsurgical techniques, complete functional recovery is rarely achieved, especially for severe peripheral nerve injuries (PNIs). Therefore, alternative therapies that can successfully repair peripheral nerves are still essential. In recent years the use of biodegradable hydrogels enriched with growth-supporting and guidance cues, cell transplantation, and biomolecular therapies have been explored for the treatment of PNIs. Bearing this in mind, the aim of this study was to assess whether Gly-Arg-Gly-Asp-Ser synthetic peptide (GRGDS)-modified gellan gum (GG) based hydrogels could foster an amenable environment for neurite/axonal growth. Additionally, strategies to further improve the rate of neurite outgrowth were also tested, namely the use of adipose tissue derived stem cells (ASCs), as well as the glial derived neurotrophic factor (GDNF). In order to increase its stability and enhance its bioactivity, the GDNF was conjugated covalently to iron oxide nanoparticles (IONPs). The impact of hydrogel modification as well as the effect of the GDNF-IONPs on ASC behavior was also screened. The results revealed that the GRGDS-GG hydrogel was able to support dorsal root ganglia (DRG)-based neurite outgrowth, which was not observed for non-modified hydrogels. Moreover, the modified hydrogels were also able to support ASCs attachment. In contrast, the presence of the GDNF-IONPs had no positive or negative impact on ASC behavior. Further experiments revealed that the presence of ASCs in the hydrogel improved axonal growth. On the other hand, GDNF-IONPs alone or combined with ASCs significantly increased neurite outgrowth from DRGs, suggesting a beneficial role of the proposed strategy for future applications in PNI regenerative medicine.

1. Introduction

The regeneration and functional recovery after a peripheral nerve injury (PNI) requires the occurrence of finely regulated events to remove the degenerated axons and myelin debris from the lesion site, so functional deficits can be compensated by regenerative mechanisms [1]. However, the recovery of gaps longer than 4 cm is minimal to non-existent [2], mainly due to extensive connective tissue scarring [3]. Autografts are the 'gold standard' procedure in clinics for the surgical repair of damaged nerves. However, this technique presents several limitations, being reported that only 40–50% of patients regain proper motor and sensorial function [4]. Therefore, there is a clear need to find strategies that overcome the autograft's drawbacks. In an attempt to ameliorate the process of nerve regeneration, tissue-engineering-based approaches are being explored. They are based on the premise that the bridges used for the reconnection of the two nerve stumps should have a more amenable microenvironment to promote the survival of the axotomized host neuronal populations, while guiding axons through the nerve gap [5]. One possible route to follow is the use of hydrogel-based biomaterials enriched with extracellular matrix (ECM) molecules, supportive cells, and exogenous factors to stimulate the different steps of regeneration. In fact, the

incorporation of ECM molecules into such biomaterials has been shown to provide adhesion sites and stimulate neurite extension *in vitro* and *in vivo* [6, 7]. Among the available hydrogels, gellan-gum (GG) is an interesting candidate for regenerative purposes because of its biocompatibility, biodegradability, and similarity to host tissues [8–11]. Indeed, we have previously shown that this biomaterial has suitable properties for nervous system repair [12]. Moreover, our lab has shown that GG modification with Gly-Arg-Gly-Asp-Ser (GRGDS) peptidic sequences improves cell adhesion, proliferation, migration, and the metabolic activity of different cell types [13, 14], including neural stem cells, olfactory ensheathing cells, and those with a mesenchymal stem cell-like phenotype, in comparison to non-modified GG.

In addition to the use of ECM-containing hydrogels for PNI treatment, the presence of exogenous factors in the peripheral nerve microenvironment appears to be crucial for nerve regeneration [15]. Their introduction in the injury site may be achieved through the transplantation of cells capable of secreting several growth factors and biomolecules that may improve the overall regeneration. Adipose tissue-derived mesenchymal stem cells (ASCs) [16] are particularly appealing for this purpose. Indeed, their secretome is known to contain high concentrations of neurotrophic factors, immunomodulatory and hematopoietic cytokines, as well as angiogenic and anti-apoptotic proteins [17] with neuroprotective activity [18, 19]. On the other hand, several growth factors have been identified as important for nerve repair by supporting axonal growth and improving resident schwann cell (SC) migration and proliferation within the damaged nerve [20]. Among these, the glial derived neurotrophic factor (GDNF) has been described as a potent promoter of neuronal survival and axonal sprouting in PNIs [20–22]. However, the use of soluble factors in these strategies is a challenge. Their short-term effect *in vivo* requires a continuous application in order to obtain a therapeutic effect [23]. In order to increase its stability and extend its bioavailability and biological activity, GDNF was conjugated covalently to iron oxide nanoparticles (IONPs) [24, 25]. Furthermore, IONPs have magnetic properties which can be used for reloading the scaffold *in vivo* with growth factor conjugated IONPs using an external magnetic field, as well as for tracking IONPs *in vivo* by MRI. Taking this into consideration, a strategy that could combine the beneficial immunomodulatory and regenerative properties of MSCs with the increased stability and delivery of growth factors provided by IONPs could represent an added value to foster the growth of injured nerves.

So far, no studies have been reported on the influence of GRGDS-GG hydrogels on neurite outgrowth. Therefore, we herein aim to first determine the ability of the modified GG-hydrogels in promoting axonal outgrowth in an *in vitro* model based on dorsal root ganglia (DRG) explants [26]. Upon this initial assessment the objective was to determine if the observed axonal outgrowth could be potentiated by co-culturing DRGs on GG-GRGDS hydrogels in the presence of ASCs and GDNF conjugated to IONPs.

2. Materials and methods

2.1. Synthesis of GDNF conjugated iron-oxide nanoparticles

Dextran-coated IONPs were prepared according to Molday and MacKenzie [26]. The GDNF (PeproTechAsia, Israel) was then conjugated covalently to the surface of the IONPs as described previously [25]. Briefly, the nanoparticles' dextran coating layer was functionalized with activated double bonds by the interaction between the hydroxyl groups of the dextran and divinyl sulfone (DVS) via the Michael addition reaction. The formed DVS-derivatized IONPs were then used for the covalent binding of the neurotrophic factor, again via the Michael addition reaction. The concentration of GDNF conjugated to the nanoparticles was determined by an appropriate ELISA Kit (PeproTech Asia, Israel).

2.2. Functionalization of GG hydrogel with GRGDS peptide

The GG hydrogel was modified with the GRGDS peptide as previously described [13]. First, GG (Sigma, USA) was modified with furan molecules (furan-GG). Briefly, GG was dissolved in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (100 mM, pH 5.5) at 37 °C. 4-(4,6-Dimethoxy-1,3,3-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Sigma, USA) and furfurylamine (Acros Organics, Belgium) were then added in a 4 : 1 molar ratio (of each reagent relative to the COOH- groups in GG) and stirred at 37 °C for 48 h. The obtained solution was dialyzed alternately against distilled water and PBS (0.1 M, pH 7.2) for 5 d. Furan-GG was then lyophilized to remove the water. The immobilization of the maleimide-containing GRGDS peptide (Mal-GRGDS, AnsSpec, USA) to furan-modified GG was performed via Diels-Alder chemistry between the GRGDS maleimide functional group and the GG furan group. To accomplish this, furan-GG was dissolved in MES buffer at 37 °C, to which Mal-GRGDS was then added in a 5 : 1 maleimide-furan molar ratio. After vigorous stirring for 48 h at 37 °C, the solution was dialyzed exactly as described above. After 5 d, water was removed from GRGDS-GG by lyophilization. As previously reported, a GG modification of approximately 300 nmol of GRGDS peptide covalently bound per milligram of GG is achieved using this protocol [13].

2.3. DRG isolation and culture on top of GRGDS-GG hydrogels

DRGs from 5 d-old neonatal Wistar-Han rat pups were dissected under sterile conditions. The DRGs

Table 1. Combination of the materials and cells used in the present experiment.

Conditions used	Cell culture system
DRG ctr	DRG culture in GRGDS-GG alone.
DRG + ASCs	Co-culture of DRGs with ASC containing GRGDS-GG hydrogels.
DRG + NPs	DRG culture in GRGDS-GG alone; GDNF-IONPs addition to the cell culture medium.
DRG + ASC + NPs	Co-culture of DRGs with ASC containing GRGDS-GG hydrogels; GDNF-IONPs addition to the cell culture medium.

were then placed on top of 50 μl drops of GRGDS-GG hydrogels and kept in culture in 24 well plates for 4 d in neurobasal medium (Gibco) supplemented with B27, L-glutamine, glucose, and 1% penicillin-streptomycin. DRG culture on top of non-modified GG was used as a control. Immunocytochemistry for neurofilament staining was performed to analyze the DRG axonal growth in the gels.

2.4. ASC Culture and GDNF iron-oxide nanoparticles cytotoxicity

The cryopreserved ASCs were a kind gift from Professor Jeff Gimble (Tulane University Center for Stem Cell Research and Regenerative Medicine and LaCell LLC (New Orleans, Louisiana, USA)). The cells were isolated according to Dubois *et al* [27] and cultured in complete α-MEM [Invitrogen, USA; supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S)] at 37 °C with 5% of CO_2. The culture medium was renewed every 2 d. Upon confluence, the cells were trypsinized and passed to new T75 flasks.

For cell counting assays, P9 ASCs were seeded in wells of 24 well plates at a cell density of 5×10^4 cell ml^{-1} and allowed to attach for 48 h in complete α-MEM at 37 °C in 5% CO_2. Then, the GDNF-conjugated NPs (10 ng ml^{-1}) were added to the cell culture medium ($n = 6$). The ASC culture in complete α-MEM without GDNF-IONPs was used as a control ($n = 6$). The cells were kept for an additional 7 d, after which the cell densities were assessed. Phalloidin (Sigma-Aldrich, USA)/DAPI (Invitrogen, USA) staining was performed and samples were analyzed by confocal microscopy (Olympus FV1200). Six representative fields per sample ($n = 6$) were chosen and analyzed at a 20 × magnification. The DAPI$^+$ cells were manually selected using Image J (NIH) software. Thereafter, the total number of cells per observed field was automatically calculated by the software and data analyzed.

2.5. Cell encapsulation within the GRGDS-GG hydrogels

In order to encapsulate ASCs in the modified gels, both GG and GRGDS-GG were dissolved in ultra-pure water to obtain a gel concentration of 1% (w/v), at 90 °C for 30 min and at 40 °C overnight, respectively. Both solutions were then mixed in a 50 : 50 GG:GRGDS-GG ratio. Calcium chloride ($CaCl_2$) was then added to the gel solution diluted 1 : 10 allowing the gelification process to occur. Finally, P9 ASCs were encapsulated in the modified-GG hydrogel at a cell density of 6×10^6cells ml^{-1}. Triplicates with 50 μl of gel each were kept in culture in a neuron medium (Invitrogen; [Neurobasal medium (Gibco) supplemented with B27, L-glutamine, glucose, and 1% Penicillin-Streptomycin]) for 4 d.

To observe the ASC morphology in GRGDS-GG hydrogels in a neuron medium, triplicates of 50 μl of each gel were kept in culture for 4 d in the referred medium supplemented with GDNF-IONPs (10 ng ml^{-1}) using Lab-Tek chambered coverglass. The ASC culture within the GRGDS-GG with a regular neuron medium was used as a control. At the end of the culture, Phalloidin/DAPI staining was performed and confocal microscopy images were taken.

2.6. DRG co-culture with ASCs and GDNF-IONPs in GRGDS-GG hydrogels

As previously described, the ASCs were encapsulated within the GRGDS-GG hydrogels at a concentration of 6×10^6 cells ml^{-1}. Four replicates of 50 μl of each were maintained in complete α-MEM for 24 h. After this time, the DRGs from 5 d-old neonatal Wistar-Han rat pups were dissected as previously described, placed on top of the ASC-containing hydrogels, and kept in culture for 4 d in neuron medium supplemented with GDNF-IONPs (10 ng ml^{-1}). The purpose of using this particular time point was to assess the effects of the tested strategy on the initial growth of new axons, and whether this could be accelerated by it. The DRG culture on top of GRGDS-GG alone, in the absence (DRGctr) or presence of GDNF-IONPs (DRG+NPs) was used as a control. Moreover, the DRG culture on top of the ASC-containing gels without GDNF-IONP supplementation (DRG+ASCs) was also used as a control, as summarized in table 1. Immunocytochemistry was performed to evaluate the DRG neurite outgrowth within the gels.

2.7. Immunocytochemistry and Phalloidin/DAPI staining

For the immunocytochemistry staining, the following antibodies were used: Mouse monoclonal anti-human neurofilament 200 kDa (Millipore) as the primary antibody and Alexa fluor 488 goat anti-mouse IgG (Invitrogen) as the secondary antibody. After 4 d of culture, the samples were fixed in a PBS

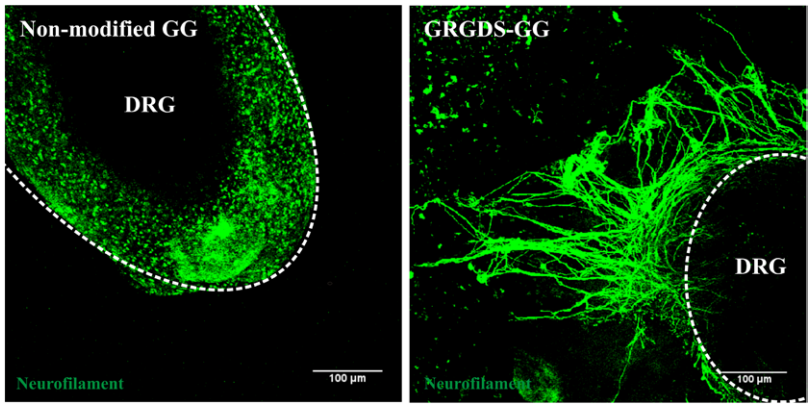

Figure 1. Axonal growth from DRG explants cultured on gellan-gum hydrogels. After 4 d of cell culture, immunocytochemistry analysis revealed that the GRGDS-GG gels promoted DRG's axonal growth, when compared to regular gels (non-modified GG).

solution containing 4% paraformaldehyde (PFA) for 45 min at room temperature (RT) and then washed with PBS. For cell membrane permeabilization, the samples were incubated with 0.3% Triton X-100 (Sigma, USA) for 10 min at RT, and then washed three times with PBS. A blocking buffer solution [PBS containing 10% fetal bovine serum (FBS)] was used for blocking for 90 min at RT, after which the samples were incubated with the primary antibody (diluted 1 : 200 in PBS solution with 10% FBS) for 48 h at 4 °C. Subsequently, the samples were again washed three times with PBS solution containing 0.5% FBS, and incubated with the secondary antibody (diluted 1 : 1000 in PBS/0.5%FBS solution) overnight at 4 °C.

For Phalloidin/DAPI staining, the cells were fixed with 4% PFA for either for 30 or 45 min (in wells or in GRGDS-GG, respectively) at RT, washed with PBS and permeabilized with 0.3% Triton X-100. After PBS washing, Phalloidin (0.1 μg ml^{-1}; Sigma) and DAPI (1 μg ml^{-1}; Invitrogen) were added to the cells for 30 or 45 min (in wells or GRGDS-GG, respectively).

2.8. DRG neurite outgrowth quantification

The confocal images of the DRG explants cultured on the GG-GRGDS hydrogels were analyzed with Image J software in order to quantify the area occupied by the neurites within the gel [28]. For this, the image scale was first set and the area occupied by the DRG itself was excluded. The images were then converted to 8 bits and binary. Through the 'analyze particles' menu, the software automatically calculated the area of neurite outgrowth, considering the image's dark background as a contrast.

2.9. Statistical analysis

The statistical analyses were performed using GraphPad Prism Software (version 5.00 for Windows). A t-student test was used for the cell counting assays, whereas a one-way ANOVA, following by a Tukey *post-hoc* multiple comparisons test, were used for the quantification of the area of neurite outgrowth. Statistical significance was defined as $p \leqslant 0.05$ for a 95% confidence level. The data were presented as mean ± standard deviation (Mean ± SD).

3. Results

3.1. DRG cultures on GRGDS-GG hydrogels

DRG explants were cultured on top of GRGDS-GG hydrogels in a neuron medium for 4 d. Immunocytochemistry for neurofilament staining was then performed, followed by confocal microscopy analysis in order to observe the axonal growth from the DRGs.

As shown in figure 1, the insertion of GRGDS peptides into GG was beneficial for neurite outgrowth. In fact, while in the modified GG-GRGDS hydrogel it is possible to observe a high degree of neurite arborization and growth, in the native form of the hydrogel none of these phenomena were witnessed.

3.2. Combination of GRGDS-GG hydrogels with ASCs and GDNF-IONPs

3.2.1. Biological effect of GRGDS-GG and GDNF-IONPs on ASCs

In order to determine the impact of the GDNF-conjugated iron nanoparticles on ASC morphology and cell densities, the latter were cultured in the presence of the NPs in both 2D (figures 2(a) and (b)) and 3D culture (figures 2(c) and (d)) systems. The ASCs were found to extend their cytoplasmic processes in both 2D and 3D environments, acquiring their typical fibroblast-like morphology [29]. Moreover, the cell densities of the ASCs (figure 2(e)) were not significantly affected by the supplementation and presence of GDNF-conjugated NPs. (ASC + NPs; 17,4 ± 3,60 DAPI$^+$ cells), when compared to the ASC culture in a regular medium (ASC ctr; 16,5 ± 1,05 DAPI$^+$ cells).

3.2.2. Co-culture of DRG explants with ASCs and GDNF-IONPs in GRGDS-GG hydrogel

The immunocytochemistry analyses on the growth of DRG neurites in the modified GG showed that the hydrogel by itself was able to support axonal growth (figure 3(a); DRG ctr). This effect was further potentiated

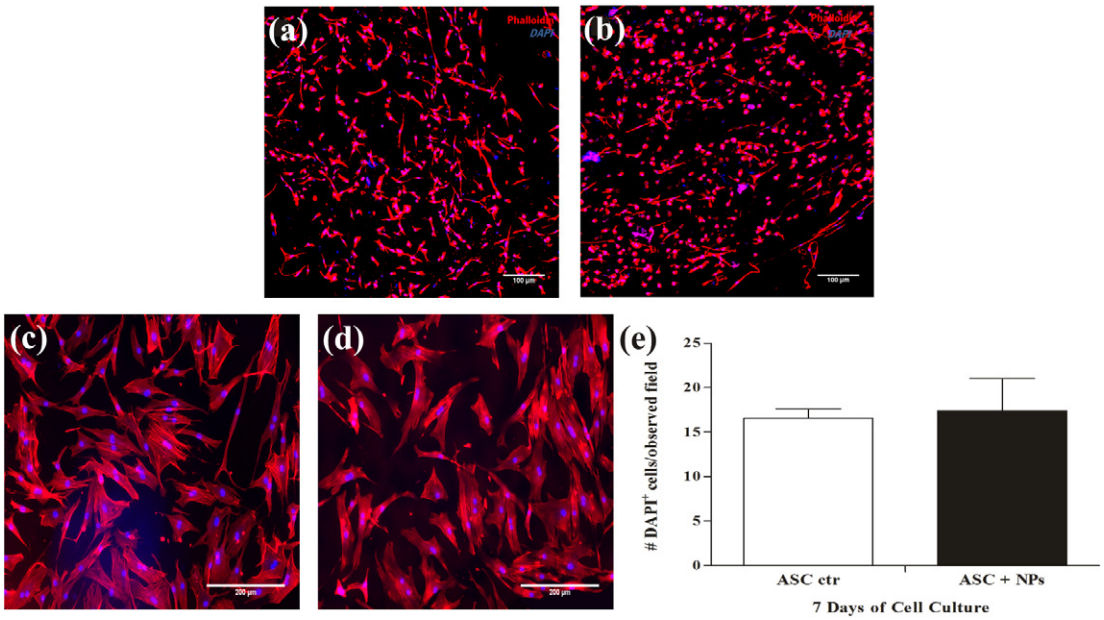

Figure 2. Morphology and density of ASCs on 2D and 3D cell culture systems. Confocal analyses revealed similar morphology of ASCs when encapsulated within the GRGDS-GG hydrogels and cultured on the 2D environment, both in the absence (a,c) or presence (b,d) of GDNF-IONPs. The cell cytoplasm was stained with phalloidin (red) and the nuclei counterstained with DAPI (blue). The cell density (e) was also found to be similar either in the absence or presence of the GDNF-IONPs. ($n = 6$; Mean ± SD).

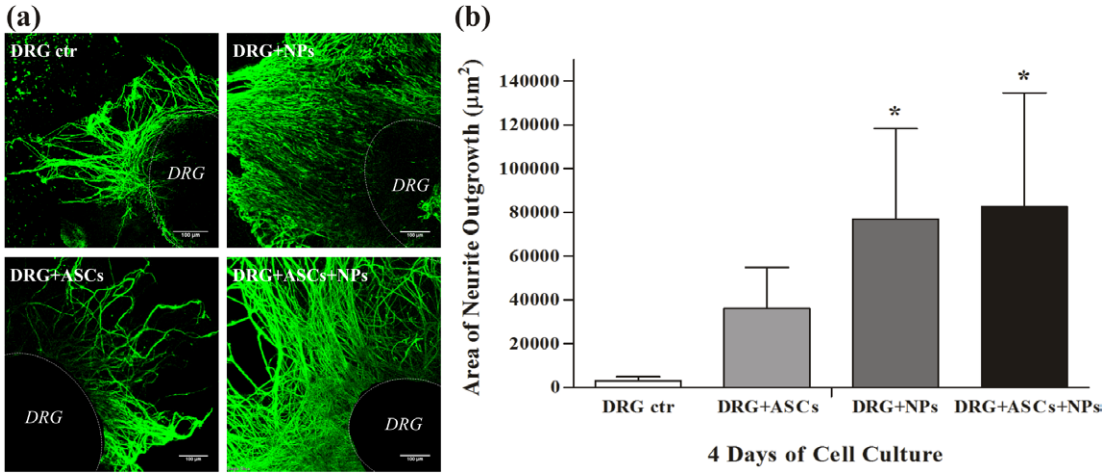

Figure 3. DRG neurite outgrowth in ASCs-containing GRGDS-GG and GDNF-NPs. (a) Immunocytochemistry analyses of the DRG culture on top of GRGDS-GG hydrogels after 4 d revealed an extensive axonal growth throughout the gel, more pronounced in the presence of GDNF-IONPs, in comparison to the DRG cultured either in GG alone (DRG ctr) and ASC-containing hydrogels (DRG + ASC). (b) Quantification of the DRG neurite outgrowth area (μm^2) was confirmed to be significantly higher in the presence of GDNF-IONPs (DRG + NPs; DRG + ASCs + NPs) versus DRG ctr. ($n = 4$; mean ± SD; * $P < 0.05$).

by co-culturing DRGs with ASCs and GDNF-IONPs (figure 3(a); DRG + ASCs; DRG + GDNF-IONPs; DRG + ASCs + GDNF-IONPs).

This qualitative analysis was further corroborated by quantifying the area of neurite outgrowth of the DRG explants in the GRGDS-GG hydrogels (figure 3(b)). The co-culture of DRGs with ASCs (DRG + ASCs) had an 11-fold increase in the area of neurite outgrowth (μm^2), in comparison with DRGs cultured in GRGDS-GG alone (DRG ctr). This indicates a positive effect of ASCs on the axonal growth, although no statistical differences were observed. The presence of the GDNF-IONPs in culture promoted a significantly higher neurite outgrowth in comparison with the control (DRG ctr) in a 24-fold (DRG + NPs) and 26-fold increase (DRG + ASC + NPs), respectively. However, there was no significant difference in the DRG neurite outgrowth between cultures treated with ASCs combined with GDNF-IONPs (DRG + ASCs + NPs) and cultures treated with GDNF-IONPs alone (DRG + NPs).

4. Discussion

The use of gellan-gum hydrogels in combination with GRGDS peptidic sequences was herein studied,

followed by their combination with ASCs and exogenous GDNF-conjugated to IONPs.

As hydrogels have been unsatisfactory in supporting nerve regeneration [30], their functionalization with ECM molecules such as GRGDS peptides could be advantageous for promoting axonal growth *in vitro*. In the present study, we demonstrated that the GRGDS-GG hydrogel by itself was able to support DRG neurite outgrowth. The addition of this peptide to other polymers such as collagen and polyethylene glycol also showed to improve neurite extension and density [7, 31]. Curiously, Ferris and colleagues [10] showed that the addition of RGD to purified GG did not significantly impact the behavior of PC12 cells. This fact could be related to the conformation of the peptide itself within the modified GG, or with the *in vitro* cell system used for this work. Although PC12 cells are a neuronal cell line, they do not disclose all the properties of the primary or explant cultures of CNS/PNS derived tissues, a fact that could impact the outcomes of such experiments. Other modified ECM-like hydrogels, such as elastin-based gels, were shown to significantly improve neurite outgrowth and the extension of DRG explants when compared to non-modified hydrogels [32]. These studies suggest that other ECM molecules may be used for GG modification, namely laminin or elastin, to improve GG performance in promoting axonal regeneration.

One of the possible existing routes to increase neurite/axonal growth within 3D hydrogels is through the use of mesenchymal-like stem cells, such as those derived from the adipose tissue (ASCs). In fact, these cells are known to have a secretome rich in immunomodulatory and hematopoietic cytokines [33], as well as angiogenic and anti-apoptotic proteins [34], and neuroregulatory molecules, some of which with a direct impact on nerve growth [17, 35]. Therefore, we set out to determine if ASCs could be cultured within the GG-GRGDS ECM-like matrix on one hand, and on the other if they could promote neurite/axonal growth. The results revealed that, in fact, GG-GRGDS-based hydrogels were able to promote ASC attachment and growth, reflecting the cell-GRGDS peptides interaction. In fact, this is in line with what has been previously reported by our and other laboratories [10, 13, 14]. For instance, Ferris and colleagues revealed that by modifying a purified version of GG with an RGD containing peptide it was possible to support the growth of C2C12 cells [10]. Regarding the effects of ASCs on axonal growth, an improvement of DRG neurite outgrowth was observed when ASCs were present in the hydrogel, although with no statistical difference. Furthermore, the use of exogenous GDNF-conjugated IONPs in the *in vitro* system had clearly improved DRG axonal growth in the GRGDS-GG gels. This effect of GDNF on axonal growth was already expected since this factor was previously described as a potent neuroprotector and neurite branching promoter and extremely important for neuronal survival and nerve regeneration following a PNI [36].

Finally, when ASCs and GDNF-IONPs were used in combination in the GRGDS-GG hydrogels, no cytotoxic effect of the NPs on ASC was observed, suggesting these two approaches are suitable for a combined therapy. Indeed, the neurite outgrowth from DRGs was significantly improved when ASCs and GDNF-IONPs were used in a cell culture system when compared to the control. However, it appears that there is no synergistic effect of the two approaches, as no significant differences were observed between the use of ASCs combined with GDNF-IONPs and GDNF-IONPs alone. This fact may be due to a higher concentration of the exogenous GDNF than the factors secreted by ASCs, therefore attributing the observed effect solely to the presence of the GDNF in the cell culture system. Nevertheless, ASCs secretome is widely described to also have a non-inflammatory [17], non-apoptotic, and angiogenic [34] character, being able to foster an environment amenable to inducing regeneration. Bearing this in mind it is possible to envisage a combined therapy in which ASCs, through their secretome, protect the injured tissue, and with it create an environment favorable for regeneration, while axonal growth will be stimulated by the GDNF bound to IONPs.

5. Conclusions

GRGDS-GG hydrogels alone were able to promote DRG neurite outgrowth. The modified gels also showed their for support ASC attachment and growth, as well as potentiated DRG axonal growth, when compared to the controls. These results revealed the importance of introducing chemical cues within hydrogels in order to obtain axonal growth for future application in PNI treatment. Similarly, the administration of GDNF-IONPs to the cell culture system also promoted a significantly higher DRG neurite outgrowth in comparison to the control. Finally, the combination of ASCs containing GRGDS-GG hydrogels and GDNF-IONPs was shown to significantly improve DRG axonal growth versus the control, without any detrimental effect of the NPs on cell behavior, although no cumulative effect of ASCs and GDNF-IONPs was observed.

According to the results obtained here, the combination of these three strategies can represent an option for PNI-related therapies. Future work will focus on the impact of GDNF-IONPs, as well as of the GRGDS peptide, on ASC secretome in order to study more closely its regenerative potential for PNI applications.

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