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Gelatin Based Hydrogel for Vascular Endothelial Growth Factor Release in Peripheral Nerve Tissue Engineering

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ABSTRACT

Hydrogels are promising materials in regenerative medicine applications due to their hydrophilicity, biocompatibility and capacity to release drugs and growth factors in a controlled manner. In this study, biocompatible and biodegradable hydrogels based on blends of natural polymers were used in *in vitro* and *ex vivo* experiments as a tool for VEGF controlled release to accelerate the nerve regeneration process. Among different candidates, the angiogenic factor VEGF was selected since angiogenesis has been long recognized as an important and necessary step during tissue repair. Recent studies pointed out that VEGF has a beneficial effect on motor neuron survival and Schwann cell vitality and proliferation. Moreover, VEGF administration can sustain and enhance the growth of regenerating peripheral nerve fibres. Hydrogel preparation process was optimized to allow VEGF functional incorporation, while preventing its degradation and denaturation. VEGF release was quantified through ELISA assay whereas released VEGF bioactivity was validated in Human Umbilical Vein Endothelial Cells (HUVEC) and in a Schwann cell line (RT4-D6P2T) by assessing VEGFR-2 and downstream effectors Akt and Erk1/2 phosphorylation. Moreover, dorsal root ganglia explants cultured on VEGF releasing hydrogels displayed increased neurite outgrowth proving confirmation that released VEGF maintained its effect, as also confirmed in tubulogenesis assay. In conclusion, a gelatin based hydrogel system for bioactive VEGF delivery was developed and characterized for its applicability in neural tissue engineering.

KEY WORDS: peripheral nerve, tissue engineering, drug releasing hydrogel, gelatin hydrogels, vascular endothelial growth factor, nerve regeneration

1. INTRODUCTION

The peripheral nervous system (PNS) is characterized by an intrinsic regenerative potential following nerve injury; nevertheless in many cases regeneration is insufficient, leading to poor functional recovery and lifelong disturbances or loss of functions mediated by the injured nerve. Moreover, development of neuropathic pain is often perceived following nerve damages. Thus, peripheral nerve injury (PNI) importantly compromises the quality of life of affected individuals and has a noteworthy socioeconomic impact (Nicholson and Verma 2004, Taylor 2006).

Traumatic PNI may lead to a gap between nerve ends. When the direct suture of proximal and distal stumps is not practicable, the current gold standard is the interposition of an autologous nerve graft. This approach has several disadvantages, such as graft availability, size mismatch, and donor site morbidity. The most important problem of grafting is that nerve regeneration is never complete. Over the past decades the development of artificial nerve guidance channels (NGCs) has therefore been of great interest (Battiston et al. 2005, Deumens et al. 2010, Schlosshauer et al. 2006, Slutsky 2005). Until now, the use of these channels has not lead to results comparable to autologous nerve grafting. A NGC should have a tridimensional structure capable of providing structural support to the newly formed tissue, allowing cell adhesion, proliferation and migration, tissue in-growth and vascularization. Several methods have been used to enhance NGC potential for nerve regeneration. Numerous modifications to NGC have been investigated to increase the length of the gap that can be bridged and to improve axonal regeneration (Giacca and Zacchigna 2012, Kempton et al. 2009, Ruiz de Almodovar et al. 2009, Zhang H. et al. 2013). These modifications include the addition of Schwann cells, the use of internal scaffolds (such as sponge, filaments,

multichannel nerve tubes and conductive polymers structures to regulate flexibility, conduit diameter dimensions and porosity degree), and the addition of growth factors (GFs) to promote axonal outgrowth, neuronal survival and Schwann cell proliferation and migration (Giacca and Zacchigna 2012, Haninec et al. 2012, Moimas et al. 2013).

An increasing number of studies pointed the attention on vascular endothelial growth factor (VEGF) as potential therapeutic in nerve repair (Giacca and Zacchigna 2012, Haninec et al. 2012, Kempton et al. 2009, Moimas et al. 2013, Pereira Lopes et al. 2011, Ruiz de Almodovar et al. 2009, Schratzberger et al. 2000, Zhang H. et al. 2013). VEGF is a potent angiogenic factor that stimulates endothelial cell migration and proliferation, blood vessel formation and vascular permeability increase (Holmes and Zachary 2005, Neufeld et al. 1999, Ruiz de Almodovar et al. 2009). VEGF gene gives rise to several isoforms by alternative splicing. The three major VEGF isoforms, consisting of 121, 165 and 189 aminoacids in humans, are named VEGF-A121, VEGF-A165, and VEGF-A189. VEGF-A121 isoform is freely diffusible, whereas VEGF-A189 binds extracellular matrix proteins, thus remaining spatially localized. VEGF-A165 isoform displays intermediate characteristics remaining partly bound to the pericellular matrix and partly diffusible. VEGF-A165 isoform binds to both tyrosine-kinase receptors VEGFR-1 (fms-like tyrosine kinase 1 or Flt1) and VEGFR-2 (kinase insert domain containing receptor, KDR in humans or fetal liver kinase 1, Flk1 in mice). VEGF-A165 also binds to both neuropilin-1 (NRP1) and neuropilin-2 (NRP2). NRPs are single transmembrane glycoproteins that may enhance VEGF signaling by acting as coreceptors of VEGF receptors. Binding of VEGF to its receptor results in receptor dimerization and phosphorylation. In particular, phosphorylation of tyrosine residue 1175 results in activation of extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt

signaling pathway, leading to positive regulation of proliferation and survival (Grunewald et al. 2010, Neufeld et al. 1999, Robinson and Stringer 2001, Ruiz de Almodovar et al. 2009). Several methods have been implemented to deliver growth factors into NGCs (de Ruiter et al. 2009, Deumens et al. 2010). The most common system for delivering growth factors is to fill the NGC lumen with carriers such as gels (des Rieux et al. 2011, Hao et al. 2007, Lee et al. 2004, Norton et al. 2005, Sun et al. 2011, Zhang W. et al. 2011), fibers (Zhang H. et al. 2013), nano-micro-particles (Cleland et al. 2001, des Rieux et al. 2011, Jay and Saltzman 2009, Shin et al. 2013, Tan et al. 2011) and fibrin-based scaffolds (Briganti et al. 2010). Ideally, these matrices can be loaded with growth factors for a controlled release for a defined time, at a therapeutic concentration. Hydrogels have long received attention because of their thixotropic and injectable nature and easiness of growth factor incorporation, which makes them trouble-free to employ as internal fillers for hollow guides. In a previous study agar/gelatin blends (A/GL), cross-linked with genipin (A/GL-GP) have been prepared with a weight ratio of 20/80 (wt./wt.) and characterized to have suitable injectable nature (Tonda-Turo et al. 2014). The prepared biomaterial allowed glial cell growth providing confirmation of its biocompatibility. Moreover, the hydrogel fabrication process has been set up in mild conditions in order to allow growth factor incorporation (body temperature, no organic solvents or presence of stabilizing additives) (Tonda-Turo et al. 2014).

In this study, the possibility to encapsulate VEGF and to obtain a controlled release of bioactive VEGF from the developed hydrogel was investigated. Different amounts of VEGF-A165 were encapsulated into A/GL-GP and the release kinetics were analyzed.

Sequential observations were carried out to evaluate the release rate and bioactivity of VEGF-A165 incorporated into the hydrogel.

2. MATERIALS AND METHODS

2.1 Hydrogel preparation and VEGF-A165 incorporation and release

Agar (A, Sigma Aldrich) was dissolved in phosphate buffered saline (PBS, Sigma Aldrich), at 90°C, to a final concentration of 0.04%. After 1 hour of incubation, 0.16% gelatin (GL, type A from porcine skin, Sigma Aldrich) was added and dissolved at 50°C for 30 minutes. The cross-linking process was performed by dissolving 0.05% genipin (Challenge Bioproducts LTD), at 50°C for 30 minutes. The resulting solution (A/GL-GP solution) was poured into a Petri dish or on glass coverslips depending on the assay to be performed and allowed to solidify O/N (overnight) at RT (room temperature). VEGF-A165 was incorporated at the end of the cross-linking step, the temperature was cooled down to 37°C (to preserve VEGF-A165 bioactivity) and recombinant Human VEGF-A165 (R&D Systems) was added at the concentration of 50, 100 and 200 ng per ml and mixed within the hydrogel solution as schematized in figure 1 A. The hydrogel preparations containing VEGF-A165 were allowed to solidify overnight at 4°C in order to preserve VEGF-A165 bioactivity. The amount of VEGF-A165 to be incorporated within the gel was chosen based on literature data (Deister and Schmidt 2006, Fu et al. 2007, Hao et al. 2007, Jay and Saltzman 2009, Pereira Lopes et al. 2011, Sondell et al. 1999, Sondell et al. 2000).

2.2 *In vitro* VEGF-A165 release kinetics

50, 100 and 200 ng of VEGF-A165 were incorporated in 1 ml of gel. The different hydrogels were poured in 35 mm diameter tissue culture dish and covered with 2.5 ml of serum free Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) and incubated at 37°C under 5% CO_2. For ELISA and Western blot quantification 0.5 and 2

ml supernatants aliquots were harvested respectively (Fig. 1 B). For tubulogenesis and DRG explants outgrowth assay 0.5 ml of M199 serum free medium and F12-BME medium were used respectively to cover different hydrogels preparations. Supernatants were collected all together up to a final volume of 15 ml (Fig. 1 B). The supernatants were retrieved and replaced with fresh medium at predetermined time intervals (1, 3, and 6 hours 1, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 34, 36, 38, 41, 43, 45, 48, 50, 52, 54, 56, 58, 61, 63, and 65 days). The amount of VEGF-A165 in the single harvested supernatants was determined using Human VEGF-A165 Immunoassay (Quantikine, R&D Systems) following the manufacturer's instruction.

2.3 Cell culture

RT4-D6P2T schwannoma cell line was purchased from American Type Culture Collection (ATCC- catalog number CRL-2768) and cultured following manufacturer's instruction.

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cord veins and cultured as previously described (Bussolino et al. 1992, di Blasio et al. 2010).

2.4 Bioactivity assay-Western blot

Both HUVEC and RT4-D6P2T cells were stimulated with 1 ml of single harvested supernatants. As negative and positive controls HUVEC or RT4-D6P2T cells were stimulated with serum free culture medium without or with 30 ng VEGF-A165/ml respectively. Protein extraction and Western blot were performed as previously described (di Blasio et al. 2010, Tonda-Turo et al. 2011) using the following primary antibodies: phospho-VEGF Receptor 2 (Tyr1175) (19A10) rabbit mAb (#2478, Cell Signaling), VEGF receptor-2 (55B11) rabbit mAb (#2479, Cell Signaling), phospho-

p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) mouse mAb (#9106, Cell Signaling), p44/42 MAPK (Erk1/2)(137F5) rabbit mAb (#4695, Cell Signaling), phospho-Akt (Ser473) (587F11) mouse mAb (#4051, Cell Signaling) and Akt rabbit mAb (#9272, Cell Signaling). All antibodies were used at a final dilution of 1:1000.

2.5 Bioactivity assay-HUVEC tubulogenesis assay

The formation of capillary-like structures was examined on growth factor-reduced matrigel® in 24 wells plate. Matrigel® was added to each coverslip at a concentration of 8 mg/ml (300 µl) and incubated at 37°C for 30 minutes to allow polymerization. HUVEC were plated at cell density of 5 x 10^4/well onto matrigel®. 1 ml of M199 serum free medium (negative control), M199 complete medium containing bovine brain extract (positive control), M199 serum free medium containing 50, 100 or 200 ng VEGF-A165/ml (positive control) and supernatants pools harvested from hydrogels containing 50, 100 or 200 ng VEGF-A165/ml of hydrogel were added to each well and refreshed twice. After 4h of incubation in 5% CO_2 humidified atmosphere at 37°C, cell organization was examined. Cells were immunostained as previously described using VEGFR-2 Rabbit mAb primary antibody (Cell Signalling, 55B11) and Cy3-conjugated goat αRb IgG (H+L) as secondary antibody (diluted 1:200 in PBS, 111-165-003, Jackson Immuno Research) and DAPI was used to stain nuclei. The length of capillary-like structures was acquired at the confocal microscope (Leica) and quantified with the imaging software ImageJ. Data were expressed as mean ± SEM.

2.6 Bioactivity assay-axonal outgrowth

In order to evaluate the bioactivity of released VEGF-A165 after its incorporation, two different assays were performed.

In the first assay DRG explants were cultured on matrigel® (Fornaro et al. 2008) and stimulated with 2 ml of conditioned medium harvested from hydrogels in which 0, 50, 100 or 200 ng VEGF-A165/ml of gel were incorporated. As negative and positive control no GFs or 50 ng of NGF /ml of medium were added according to literature data (Deister and Schmidt 2006, Gorokhova et al. 2014). Conditioned medium used in this assay was F12-BME medium. DRGs explants were harvested from adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190-220g. A total of six ganglia were mounted per coverslips. A total of 9 rats have been used in order to perform experiments in technical and biological triplicate. Rats were sacrificed by a lethal i.m. injection of tiletamine + zoletil. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC). Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

In the second assay, DRG explants were cultured on hydrogel or matrigel® in which no GFs (negative control), or 50 ng NGF /ml of hydrogel (positive control) or 50, 100 or 200 ng VEGF-A165/ml of hydrogel were incorporated by adding the GFs directly to the gel solution before the polymerization step as described above. Ganglia were mounted on coverslips coated with 100 μl matrigel® or hydrogel, containing or not the GFs. A total of six ganglia were mounted per coverslips. A total of 18 rats have been used in order to perform experiments in technical and biological triplicate.After 30 minutes incubation at 37°C, to allow matrigel® or hydrogel polymerization, 2 ml of complete F12-BME medium (Gibco) were added.

After 48h incubation, explants were fixed with 4% paraformaldehyde (PFA) for 20 minutes at RT. Immunocytochemistry, to stain neurite, was performed using β-tubulin mouse mAb (diluted 1:1000, T8328, Sigma) and AlexaFluor488 goat αMs IgG (H+L) diluted 1:200 (A11029, Invitrogen). Nuclei were stained with 4',6-diamidino-2phenylindole (diluted 1:1 000, Sigma Aldrinch). Samples were observed with a Nikon Eclipse E800 epifluorescence microscope under appropriate filters and a Leica TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany). For quantification, the whole explants were acquired through an optical video-confocal microscope (Nikon Eclipse 80i) and the supporting software Image ProPlus (Media Cybernetics USA). Image analysis was performed by applying a threshold, in order to discriminate between white background pixels and black pixels corresponding to areas covered by neurites, thus obtaining a binary image. Three different measurements were taken. First, area occupied by axons was normalized to the area occupied by the DRG body; second, number of pixel occupied by axons and; third axons length (Gilardino et al. 2009, Zamburlin et al. 2006).

2.7 Statistics

All the experiments were performed in triplicate. Data were expressed as mean ± SEM. Statistical analysis was carried out using single-factor analysis of variance (ANOVA) post hoc Bonferroni. Values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ were considered as statistically significant.

3. RESULTS

3.1 VEGF-A165 was successfully released from the hydrogel.

Gelatin-based hydrogels containing 50, 100 or 200 ng VEGF-A165 per ml of gel solution were prepared and the release behavior of proteins was monitored *in vitro* every 2-3 days up to 65 days (Fig. 2). The capability of gelatin-based hydrogel to release VEGF-A165 was quantified by ELISA immunoassay. VEGF-A165 release was monitored until it was possible to quantify it through ELISA. After 65 days it was not possible to detect VEGF-A165 protein in the harvested supernatants. There was a VEGF-A165 burst release up to 20 days of *in vitro* culture (Fig. 2). After 20 days the amount of released VEGF-A165 becomes lower depending on the amount of initial incorporated VEGF-A165 (Fig. 2A). VEGF-A165 was released up to 58, 63 and 65 days *in vitro* from the 50, 100 and 200 ng VEGF-A165/ml hydrogel respectively. After these time points was not possible to detect the VEGF-A165 protein in the harvested supernatants through ELISA (Fig. 2A). From day 1 to day 20 incorporated VEGF-A165 was released by the hydrogel at a rate of 0.96 ± 0.11 ng (from 50 ng VEGF-A165/ ml gel), 1.85 ± 0.19 ng (from 100 ng VEGF-A165/ml gel) and 3.69 ± 0.36 ng (from 200 ng VEGF-A165/ml gel). From day 20 to day 43 the releasing rate decreases 5 times and incorporated VEGF-A165 was released by the hydrogel at a rate of 0.21 ± 0.03 ng (from 50 ng VEGF-A165/ml gel), 0.36 ± 0.07 ng (from 100 ng VEGF-A165/ml gel) and 0.86 ± 0.17 ng (from 200 ng/ml gel). From day 43 to day 65 incorporated VEGF-A165 was released by the hydrogel at a rate of 29.76 ± 10.35 pg (from 50 ng VEGF-A165/ml gel), 41.73 ± 4.78 pg (from 100 ng VEGF-A165/ml gel) and 162.49 ± 21.20 pg (from 200 ng

VEGF-A165/ml gel) (Fig. 2A). The VEGF-A165 releasing rate SEM is low and stable in the first two releasing phases (from day 1 to day 43). The higher SEM observed in the third releasing phase (from day 43 to day 65) might be due to the heterogeneous degradation and releasing rate of the different hydrogel preparations overtime. The total amount of released VEGF-A165 was 13.89 ± 0.04 ng (from 50 ng VEGF-A165/ml gel), 26.28 ± 0.14 ng (from 100 ng VEGF-A165/ml gel) and 54.55 ± 1.03 ng (from 200 ng VEGF-A165/ml gel); when the amount of released molecule is expressed as percentage of the total amount of incorporated molecule, it results to be 27.77 % ±0.08 (from 50 ng VEGF-A165/ml gel), 26.28 % ± 0,14 (from 100 ng VEGF-A165/ml gel) and 27.28 % ± 0.51 (from 200 ng VEGF-A165/ml gel) (Table 1).

3.2 VEGF-A165 is released from hydrogel and maintains its ability to activate VEGFR-2, Erk1/2 and Akt phosphorylation.

VEGF-A165 bioactivity was evaluated by stimulating HUVEC with the harvested medium as described in materials and methods section. Stimulation of HUVEC with the harvested supernatants resulted in VEGFR-2 phosphorylation leading to Akt and Erk pathways activation. Figure 3 reports the western blot analysis of the phosphorylated and total VEGFR-2, Erk1/2 and Akt proteins and the relative quantification of the level of phosphorylation for each protein - normalized to the total protein - for the three different VEGF-A165 concentrations used. VEGF-A165 present in the harvested supernatants induces VEGFR-2 phosphorylation up to 50 days for all the tested concentrations. VEGFR-2 phosphorylation level is high up to 41, 43 and 48 days when cells are stimulated with supernatants harvested from 50, 100 and 200 ng VEGF-A165/ml hydrogel respectively, then it starts to decrease. Akt and Erk-1/2 are phosphorylated up to 65 days. Erk-1/2 phosphorylation level is high up to 29 days,

when cells are stimulated with supernatants harvested from 50 and 100 ng VEGF-A165 /ml hydrogel and 38 days when cells are stimulated with supernatants harvested from 200 ng VEGF-A165/ml hydrogel, then it starts to decrease. Akt phosphorylation level does not change overtime for the three different tested conditions. The amount of VEGFR-2, Akt and Erk1-2 protein phosphorilation (Fig. 3) is strictly correlated to the total amount of released VEGF-A165 detected with the ELISA assay (Fig. 2A) resulting in a phosphorilation decrease overtime (Fig. 3).

VEGF-A165 released from the hydrogel leads to Erk1/2 and Akt phosphorylation in Schwann cells. In order to evaluate whether VEGF-A165 released from the hydrogel can exerts a biologic effect on Schwann cells, a western blot analysis on proteins extracted from RT4-D6P2T cells stimulated with VEGF-A165 was performed. Although RT4-D6P2T show low VEGFR-2 mRNA and protein expression (unpublished data) VEGF-A165 stimulation results in Erk-1/2 and Akt specific activation. Indeed stimulation of RT4-D6P2T cells with supernatants harvested from hydrogels containing different VEGF-A165 amounts resulted in phosphorylation and activation of Akt and Erk (data no shown).

3.3 VEGF-A165 released from hydrogel maintains its angiogenic effect

Tubulogenesis assay was performed on HUVEC plated onto matrigel® and stimulated with a pool of supernatants harvested from hydrogels containing different amounts of VEGF-A165. HUVEC capillary-like structure formation after VEGF-A165 stimulation was evaluated.

Results show that the formation of capillary-like structures occurred both when cells are stimulated with culture medium supplemented with 50, 100 or 200 ng VEGF-A165/ml

or with the pool of supernatants, collected from hydrogel containing the different amounts of VEGF-A165 (Fig. 4 A-H). There is no significant difference between tube length of HUVEC stimulated with the pool of supernatants collected from the hydrogel containing 50 ng/ml VEGF-A165 and HUVEC stimulated with 50 ng/ml VEGF-A165 medium. Similar results were obtained with the supernatants containing 100 and 200 ng/ml VEGF-A165. There is no difference in tubes length among the three hydrogel conditions, whereas tube length increases when cells are stimulated with VEGF-A165 at a concentration of both 100 and 200 ng/ml in comparison with 50 ng/ml (Fig. 4 I).

3.4 VEGF-A165 released from hydrogel promotes axon outgrowth.

The biologic effect of released VEGF-A165 was tested on DRG explants cultured on matrigel®. Negative and positive control conditions were performed by culturing DRG explants without or with NGF stimulation at a final concentration of 50 ng/ml (Fig. 5 A and B, respectively). DRGs were stimulated with supernatant pool harvested from hydrogels containing different VEGF-A165 concentrations (Fig. 5 C-E). Confocal pictures show that VEGF-A165 released from the hydrogel (harvested as pool) induced neurite outgrowth. Neurite density, sprouting area and neurite length were quantified (Fig. 5 F-H). DRG stimulation with supernatant pool from hydrogel containing 200 ng VEGF-A165/ml results in higher neurite density, sprouting area and neurite length compared to hydrogel containing 50 and 100 ng VEGF-A165/ml. There are no significant differences in neurite outgrowth of DRG stimulated with supernatants harvested from hydrogels containing 50 ng NGF /ml or 200 ng VEGF-A165/ml.

3.5 Gelatin-based hydrogel containing VEGF-A165 allows axon outgrowth

In order to further evaluate VEGF-A165 release and bioactivity, DRG explants were cultured on both matrigel® or hydrogel containing different amounts of VEGF-A165 (50, 100 or 200 ng/ml).

Negative and positive control conditions were performed by culturing DRG explants on matrigel® or hydrogel without or with 50 ng NGF /ml respectively (Fig.6 A and B). DRGs cultured on gels containing NGF and VEGF-A165 result in neurite sprouting (Fig.6 C-J). Neurite density, sprouting area and neurite length were measured (Fig.6 K-M).

NGF incorporation in both matrigel® and hydrogel results in higher neurite density in comparison with all the different amounts of incorporated VEGF-A165. Incorporation of 200 ng VEGF-A165/ml in both matrigel® and hydrogel results in higher neurite density in comparison with 50 and 100 ng VEGF-A165/ml incorporation (Fig.6 K).

Incorporation of 50 ng NGF /ml and 200 ng VEGF-A165/ml results in higher neurite sprouting area in comparison with 50 and 100 ng VEGF-A165/ml for both matrigel® and hydrogel condition (Fig. 6 L).

NGF incorporation in both matrigel® and hydrogel results in higher neurite length in comparison with all the different amounts of incorporated VEGF-A165. Incorporation of 200 ng VEGF-A165/ml in matrigel® results in higher neurite length in comparison with 50 and 100 ng VEGF-A165/ml incorporation and 200 ng VEGF-A165/ml incorporation in the hydrogel (Fig. 6 M).

4. DISCUSSION

The peripheral nervous system is characterized by an intrinsic potential for regeneration. Long gap nerve injuries can be successfully repaired using the autograft technique. Since this technique has some disadvantages, NGCs can be applied as artificial graft to repair nerve injuries. In this context, the presence of an appropriate microenvironment, providing molecular cues such as growth factors, plays a crucial role in nerve regeneration process.

In this work, VEGF-A165 was selected for its angiogenic and neuroprotective properties (Rosenstein et al. 2010, Rosenstein et al. 2003, Storkebaum et al. 2004). In recent years, more attention has been given to the potential role of VEGF in the nervous system suggesting its involvement in neuroprotection (Beazley-Long et al. 2013, Mohammadi et al. 2013, Rosenstein et al. 2003, Silverman et al. 1999, Yue et al. 2014). It has been reported that VEGF exerts a trophic effect on primary cortical neurons and dorsal root ganglia (DRG) by promoting neurite outgrowth (Jin et al. 2006, Rosenstein et al. 2003, Silverman et al. 1999, Sondell et al. 1999, Sondell et al. 2000). VEGF also increases neuron and satellite cell survival, and Schwann cell proliferation rate (Sondell et al. 1999). Moreover, VEGF may induce Schwann cell migration (Schratzberger et al. 2000) and microglial cell migration and proliferation (Forstreuter et al. 2002). Finally, few studies reported that the delivery of VEGF through plasmid injection (Pereira Lopes et al. 2011), transfected stem cells (Kempton et al. 2009), VEGF releasing nerve graft (Sondell et al. 1999) or VEGF releasing matrigel® filler (Hobson 2002, Hobson et al. 2000) enhance nerve regeneration. The major problem in systemic VEGF delivery is its short circulation half-life (Fu et al. 2007), due to the binding of VEGF to unspecific substrates and low stability and leading to a high degradation rate and consequently

poor effect. Actually, relatively high VEGF local concentrations are required to have a biological effect.

The major advantage of the developed gelatin-based hydrogel system is to guarantee VEGF-A165 controlled release and bioactivity. Moreover, the injectable properties of the gelatin-based hydrogel are beneficial in nerve tissue engineering application, since it can be easily used to fill a tube cavity during surgery.

The developed gelatin-based hydrogel displayed a permeable three-dimensional structure allowing a sustained release of VEGF-A165 release up to 65 days. The gelatin-based hydrogel displays the same release kinetics independently of the initial amount of incorporated VEGF-A165, reaching a 27% release percentage. The release kinetics displayed a multi-time deliveries that can be divided in three phases depending on the amount of released VEGF-A165. High VEGF-A165 release rate was displayed up to 20 days, then the amount of released VEGF-A165 decreased over time. Increasing the amount of incorporated VEGF-A165 results in longer releasing rate.

The initial VEGF-A165 burst release might come from the instauration of electrical interactions between the gelatine polymer and the VEGF-A165 molecule. The lower and controlled release rate observed after 20 days *in vitro* might be due to the instauration of stronger interaction (i.e. covalent interaction) between the gelatine polymer and the VEGF-A165 with might stabilize the growth factor structure within the hydrogel overtime leading to a long term bioactivity and controlled release.

As discussed the gelatine-based hydrogel do not release more than 27% of the initial amount of incorporated VEGF-A165, this might be due to the non complete hydrogel degradation at 58-65 days *in vitro* resulting in retirement of the growth factor.

Moreover, the instauration of covalent interaction between the gelatine polymer and the VEGF might not allow the complete VEGF release until complete hydrogel degradation occurs. Another hypothesis is that the incorporation process leads to a partially denaturation and/or degradation of the initially incorporated VEGF leading to a reduction of the growth factors releasing rate.

The bioactivity of growth factors released from a biomaterial is of critical importance to allow proper tissue regeneration. VEGF-A165 was released in a bioactive form, from the gelatin-based hydrogel, leading to VEGFR-2, Erk-1/2 and Akt phosphorylation up to 65 days *in vitro* in HUVEC cells. VEGFR-2, Erk-1/2 and Akt phosphorylation levels were high up to 20 days and then these began to decrease, depending on the amount of released VEGF-A165, being active up to 65 days according to ELISA results. The tested gelatin-based hydrogel leads to longer VEGF-A165 delivery and bioactivity in comparison with carrier system described by other authors (Ennett et al. 2006, Golub et al. 2010, Parajo et al. 2010). Furthermore, the gelatin-based hydrogel preparation method is simple and convenient for VEGF-A165 incorporation, avoiding its denaturation and degradation as demonstrated with the western blot analysis on RT4-D6PT and HUVEC cells, tubulogenesis assay and DRG explants axons outgrowth assay. The total VEGF-A165 degradation or denaturation following its incorporation into the gelatine-based hydrogel would not lead to VEGFR-2, Akt and Erk1-2 phosphorylation and capillary-like structure formation in HUVEC cells or axonal sprouting in DRGs explants following stimulation with the harvested supernatants. Altogether, these data provide evidence about the optimization of the hydrogel preparation protocol to allow functional VEGF-A165 incorporation avoiding VEGF-A165 degradation and denaturation.

Concerning the angiogenic effect of VEGF-A165, the released VEGF-A165 induced capillary-like structure formation from HUVEC cells seeded on Matrigel®. Increasing the amount of incorporated VEGF-A165 results in increased tube length, whereas conditioned medium from hydrogel containing different amounts of VEGF-A165 does not affect capillary-like tube length. These data suggest that the gelatin-based hydrogel system allows a slow and controlled VEGF-A165 release.

Bioactivity of released VEGF-A165 was evaluated using Schwann cells and DRG explants since they represent an *in vitro* model of the two main cell components involved in peripheral nerve regeneration process: glia and motor neurons. Following stimulation of Schwann cells with conditioned medium, Erk-1/2 and Akt displayed a phosphorylation trend similar to the one observed in HUVEC cells. Moreover, pool of conditioned medium induced neurite outgrowth from DRG explants; neurite density, sprouting area and length showed a proportional increase depending on the amount of incorporated VEGF-A165. Conditioned medium harvested from the hydrogel containing 200 ng VEGF-A165/ml resulted in a neurite outgrowth trend similar to NGF condition. DRG explants cultured on matrigel® or hydrogel containing NGF or VEGF-A165 result in neurite outgrowth. These data provide confirmation of the successful VEGF-A165 incorporation and release from the gelatin-based hydrogel and its bioactivity effect on DRG explants. Moreover, these results show that DRG can be successfully cultured on the gelatin-based hydrogel providing confirmation of its biocompatibility.

The characterized gelatin-based hydrogel system can be injected into empty nerve guidance channels. The resulting device can be used to bridge a gap in peripheral nerves following an injury in order to improve peripheral nerve regeneration process.

Further *in vivo* experiments will be performed in order to investigate the potential application of the VEGF-A165 loaded gelatin-based hydrogel system in comparison to a non-loaded gelatin-based hydrogel system in rat median nerve injured model.

5. CONCLUSION

The gelatin-based hydrogel system can be a useful vehicle to encapsulate and slowly release VEGF-A165 in a controlled manner overtime. *In vitro* results showed that gelatin-based drug releasing hydrogel can be suitable for bioactive VEGF-A165 release, inducing capillary-like tube formation and axonal outgrowth *ex vivo*. Growth factor delivery through the gelatin-based hydrogel provides multi-time deliveries that can be suitable for peripheral nerve regeneration application. In particular, a high release rate within the first 20 days from nerve injury may promote angiogenesis, stimulate Schwann cell migration and increase the number of re-growing axons, thus enhancing the regeneration process.

6. ACKNOWLEDGEMENTS

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LIST OF FIGURES AND TABLE CAPTIONS

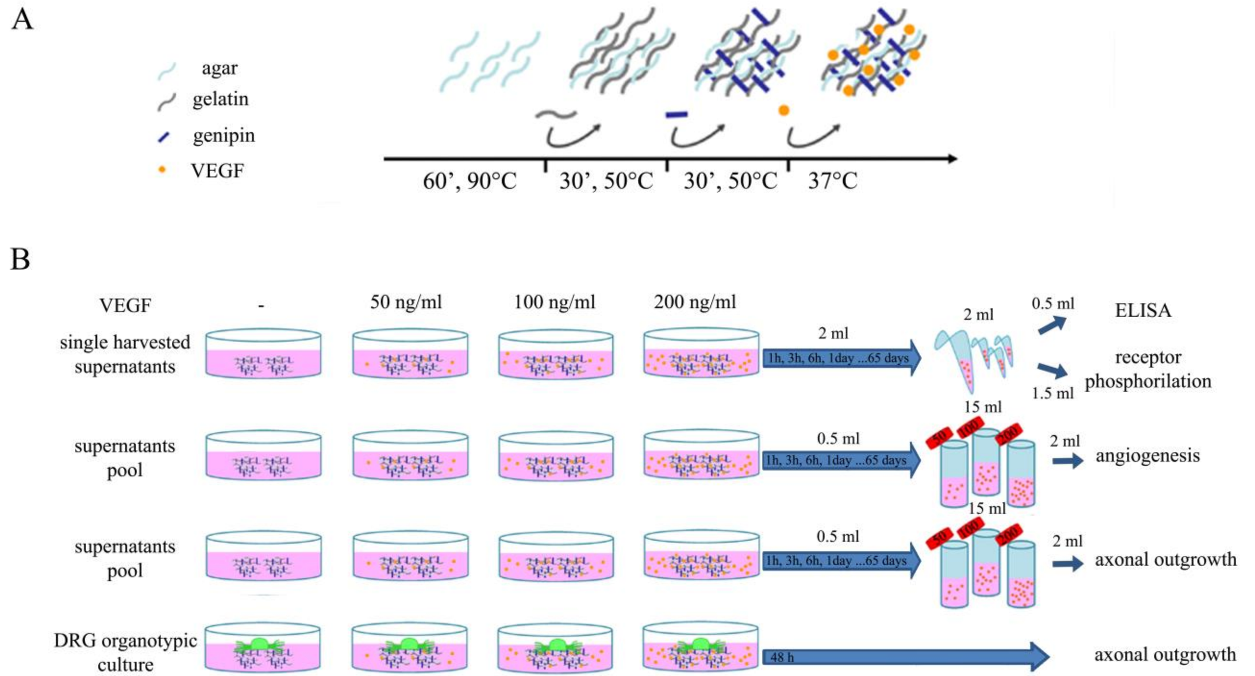


Figure 1. Experimental set up. Schematization of VEGF incorporation procedure into the gelatin-agar hydrogel (A). Harvesting medium procedure schematization (B).

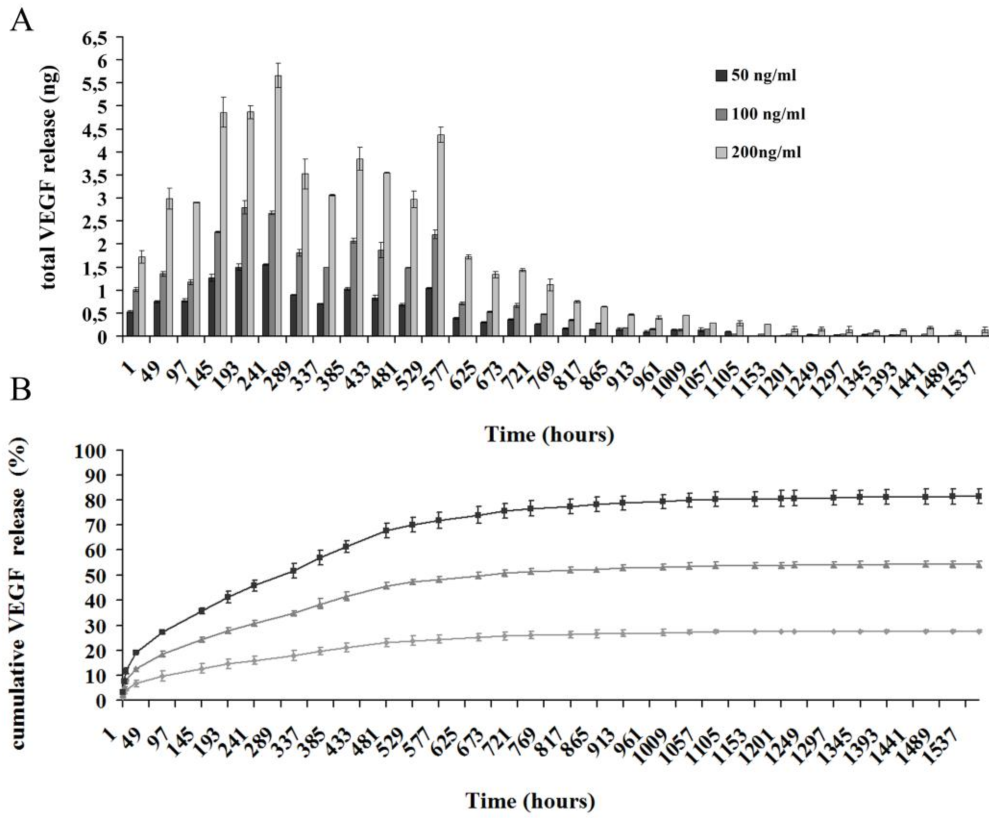
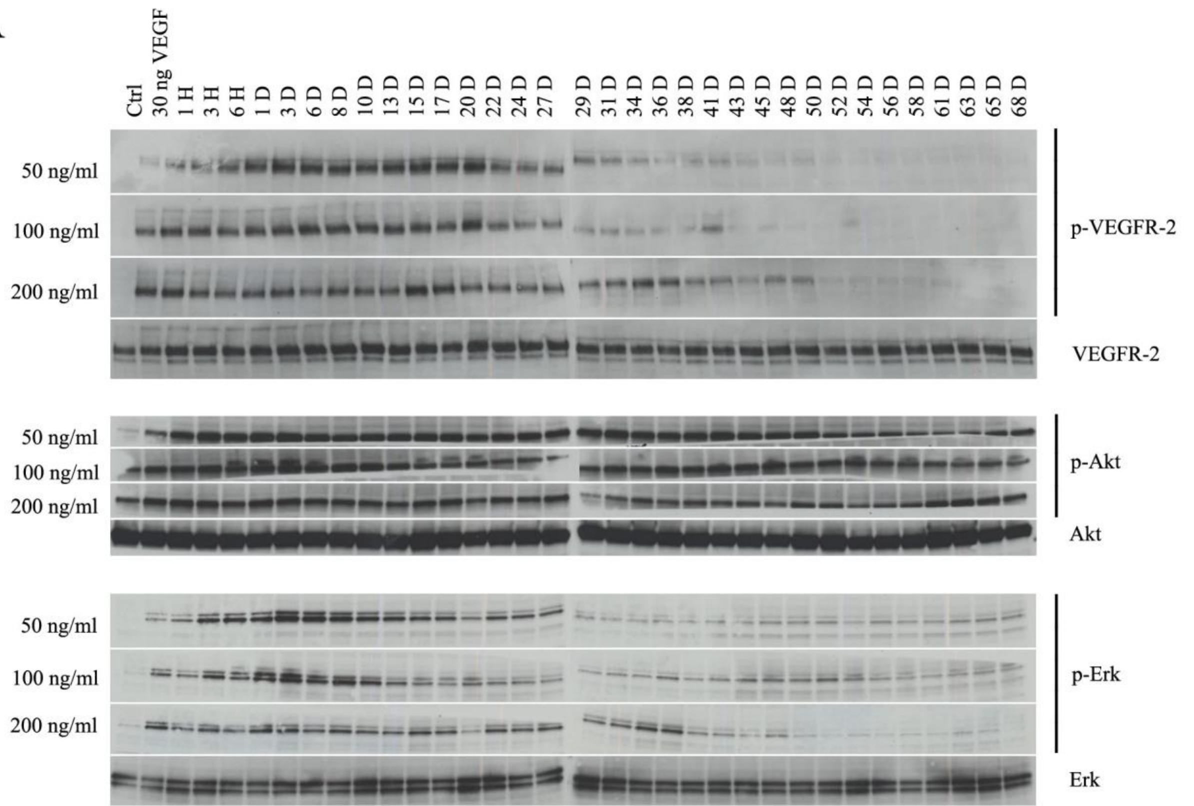


Figure 2. VEGF-A165 releasing rate from gelatin-based hydrogel. Total (A) and cumulative (B) amount of VEGF-A165 released from gelatin hydrogels incorporated with 50 (black), 100 (dark gray) and 200 ng (light gray) VEGF-A165 per ml of gel solution. Data are expressed as mean ± SEM.

A



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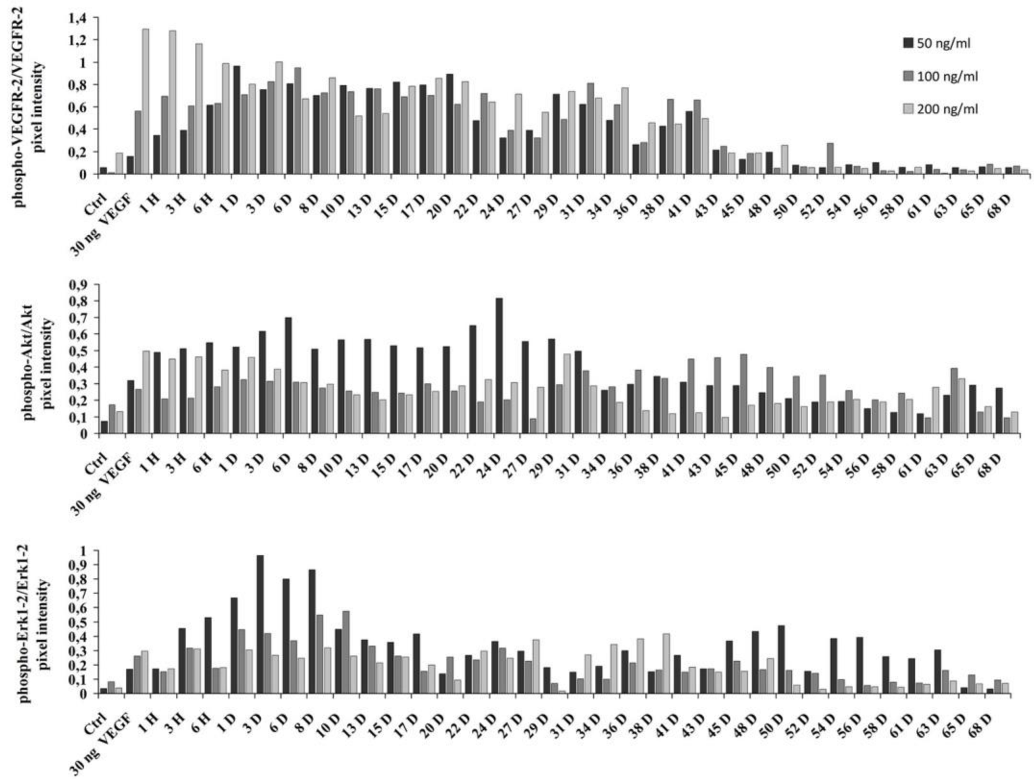

Figure 3. HUVEC stimulation with supernatants harvested from gelatin-based hydrogel containing different amounts of VEGF-A165. Western blots of phospho-VEGFR-2, VEGFR2, phospho-Erk-1/2, Erk-1/2, phospho-Akt and Akt after HUVEC stimulation with supernatants harvested from gelatin-based hydrogel containing 50, 100 or 200 ng/ml of VEGF-A165/ml (A). Phospho-VEGFR-2/VEGFR, phospho-Akt/Akt and phospho-Erk-1/2/Erk-1/2 relative quantification after stimulation of HUVEC with supernatants harvested from gelatin-based hydrogel containing 50 (black), 100 (dark gray) or 200 (light gray) ng VEGF-A165/ml(B).

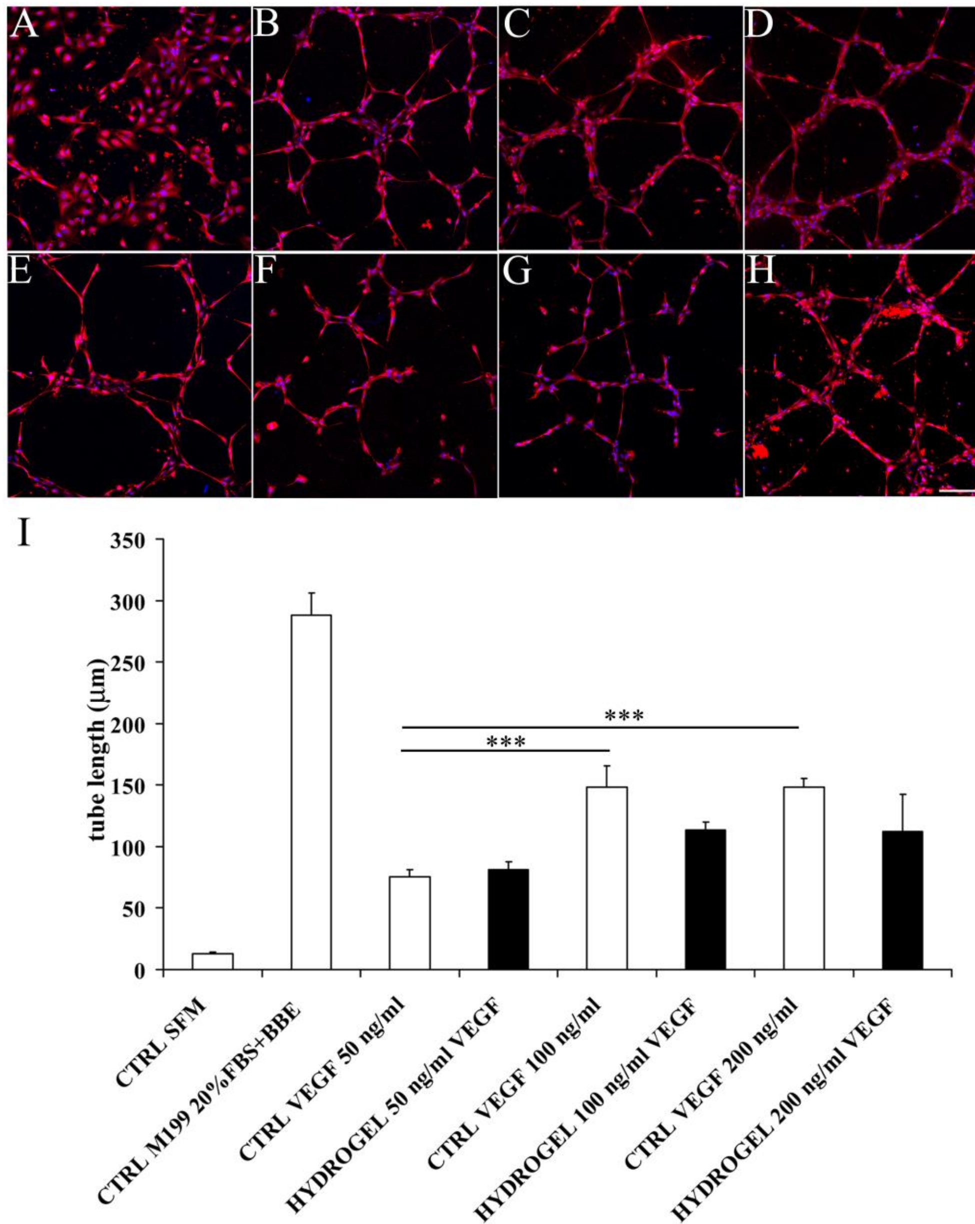

Figure 4. HUVEC tubulization assay. Representative images of HUVEC organization. Negative control condition (A), positive control condition (B), positive control condition performed by stimulating cells with 50 (C), 100 (D) and 200 (E) ng VEGF-A165/ml, stimulation with a pool of supernatants harvested from hydrogels containing 50 (F), 100 (G) and 200 (H) ng VEGF-A165/ml. Quantification of tube length (I). Data are expressed as mean ± SEM. Scale bar 100 µm.

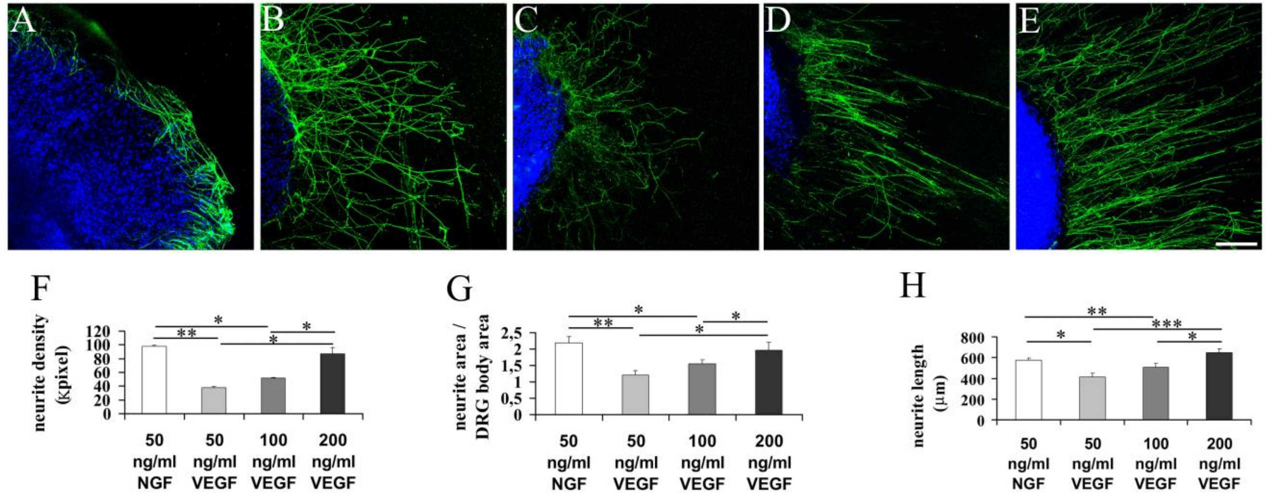

Figure 5. DRG explants stimulation with pool of supernatants harvested from gelatin-based hydrogel containing different amount of VEGF-A165. Negative control (A); positive control (50 ng NGF/ml (B); explants stimulated with a pool of supernatants harvested from hydrogels containing 50 (C), 100 (D) and 200 (E) ng VEGF-A165/ml of gel. Quantification of neurite density (F), sprouting area (G) and neurite length (H). Data are expressed as mean ± SEM. Scale bar 100 µm.

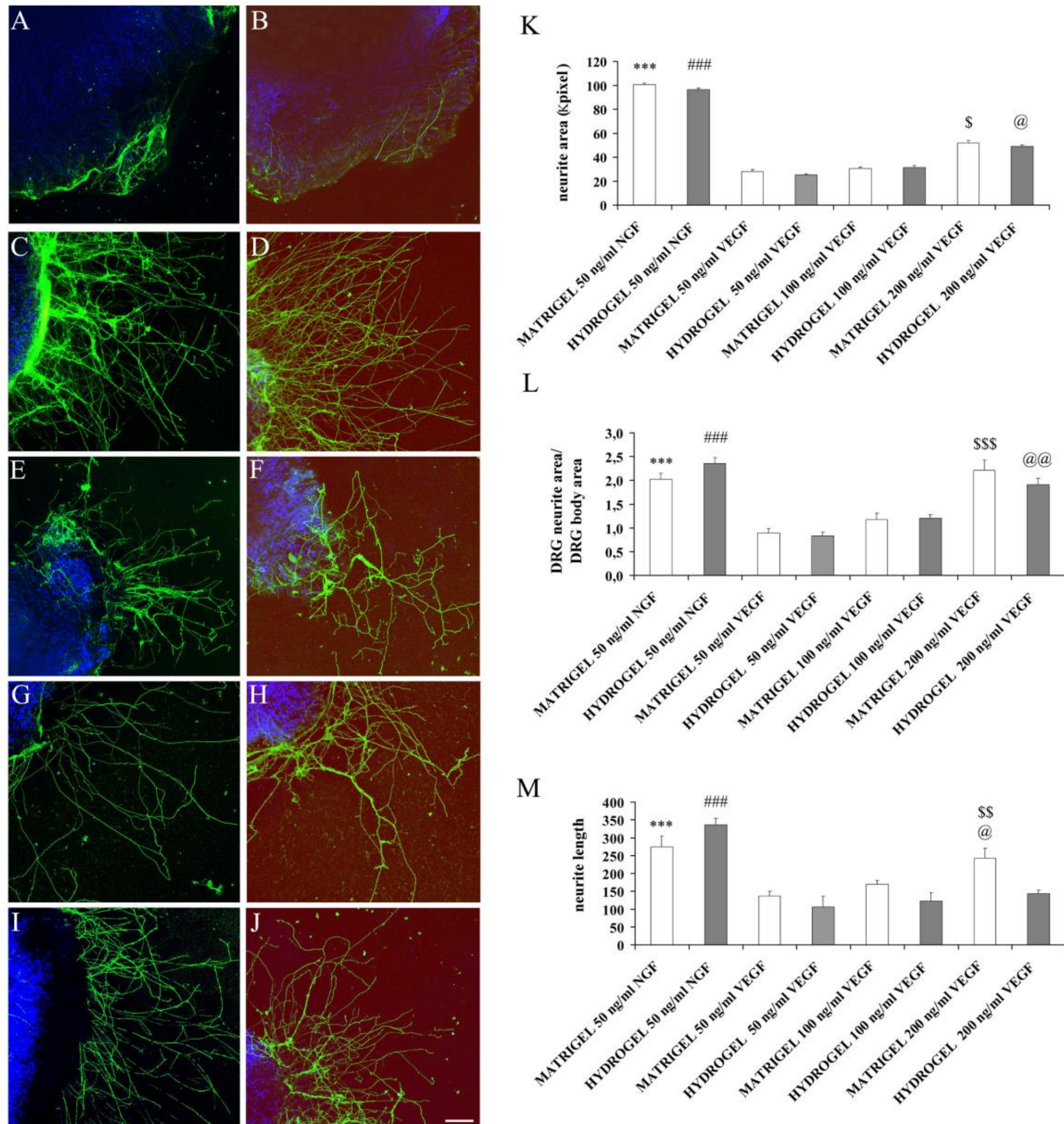

Figure 6. DRG explants cultured on matrigel® and hydrogel containing different amounts of VEGF-A165. Negative control (A and B) and positive control (50 ng NGF /ml) (C and D) performed on both matrigel® (A and C) and hydrogel (B and D); explants cultured on matrigel® containing 50, 100 or 200 ng VEGF-A165/ml (E, G, I); explants cultured on hydrogel containing 50, 100 or 200 ng VEGF-A165/ml (F, H, J). Hydrogel appears in red due to genipin auto-fluorescence. Evaluation of neurite density (K); sprouting area (L) and neurite length (M). In plot K the *** and ### refer to the

statistical difference between matrigel® and hydrogel containing respectively 50 ng NGF/ml and all the VEGF-A165 conditions. $ and @ refer to statistical differences between matrigel® and hydrogel containing 200 ng VEGF-A165/ml and all the other VEGF-A165 condition. In plot L, *** and ### refer to the statistical difference between matrigel® and hydrogel containing 50 ng NGF /ml with matrigel® and hydrogel containing 50 and 100 ng VEGF-A165/ml. $$$ and @@ refer to the statistical difference between matrigel® and hydrogel containing 200 ng NGF /ml with matrigel® and hydrogel containing 50 and 100 ng VEGF-A165/ml. In plot M, *** and ### refer to the statistical difference between hydrogel containing 50 ng NGF /ml and all the VEGF-A165 conditions. $$ and @ refer to statistical difference between matrigel® containing 200 ng VEGF-A165/ml and all the VEGF-A165 incorporated hydrogel or matrigel®. Data are expressed as mean ± SEM. Scale bar 100 µm.

	50 ngVEGF-A165/ml	100 ngVEGF-A165/ml	200 ngVEGF-A165/ml
release rate day 1-20 (ng)	0.96 ± 0.11	1.85 ± 0.19	3.69 ± 0.36
release rate day 20-43 (ng)	0.21 ± 0.03	0.36 ± 0.07	0. 86 ± 0.17
release rate day 43-65 (pg)	29.76 ± 10.35	41.73 ± 4.78	162.49 ± 21.20
total release (ng)	13.89 ± 0.04	26.28 ± 0.14	54.55 ± 1.03
% release	27.77 ± 0.08	26.28 ± 0.14	27.28 ± 0.51
total days of release	58	63	65

Table 1. The release rate, total amount of released VEGF-A165, percentage of release and total days of release from the different hydrogels containg 50, 100 and 200 ng of VEGF-A165.

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