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VEGF and FGF-2 delivery from spinal cord bridges to enhance angiogenesis following injury

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Abstract

The host response to spinal cord injury can lead to an ischemic environment that can induce cell death and limits cell transplantation approaches to promote spinal cord regeneration. Spinal cord bridges that provide a localized and sustained release of VEGF and FGF-2 were investigated for their ability to promote angiogenesis and nerve growth within the injury. Bridges were fabricated by fusion of poly(lactide-co-glycolide) microspheres using a gas foaming/particulate leaching technique, and proteins were incorporated by encapsulation into the microspheres and/or mixing with the microspheres before foaming. Compared to the mixing method, encapsulation reduced the losses during leaching and had a slower protein release, while VEGF was released more rapidly than FGF-2. *In vivo* implantation of bridges loaded with VEGF enhanced the levels of VEGF within the injury at one week, and bridges releasing VEGF and FGF-2 increased the infiltration of endothelial cells and the formation of blood vessel at 6 weeks post implantation. Additionally, substantial neurofilament staining was observed within the bridge; however, no significant difference was observed between bridges with or without protein. Bridges releasing angiogenic factors may provide an approach to overcome an ischemic environment that limits regeneration and cell transplantation based approaches.

Keywords

Spinal cord injury; angiogenesis; spinal cord bridges; PLG; protein delivery

Introduction

Drug delivery strategies are increasingly being investigated to address the limited regeneration that occurs following spinal cord injury. Spinal cord injury is characterized by an acute phase, which includes hemorrhage, the destruction of the blood brain barrier, and infiltration of inflammatory cells, followed by a sub-acute and chronic phase, which consists of secondary injury distinguished by the Wallerian axonal degeneration, and the formation of a cavity and glial scar surrounding this cavity [1]. The lack of neurotrophic factors, ischemia resulting from insufficient blood flow, and the presence of inhibiting factors, all contribute to an unfavorable microenvironment for spinal cord regeneration. Several

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neurotrophic factors have been investigated to promote neuronal survival and axonal regrowth through the injury site [2–4]. The delivery of these factors has typically been achieved using direct injection, osmotic pumps, or transplantation of genetically engineered cells. More recently, bridges have been implanted into spinal cord injuries to stabilize the injury site, promote cell infiltration, and limit cyst or cavity formation. These bridges have the potential to be used as vehicles for the sustained release of factors to target one or more processes that limit regeneration [5–11].

Angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2), have been proposed to address ischemia following injury, and were implicated in neuroprotection [1,12] and/or nerve regeneration [2,4,13,14] after spinal cord injury. The ischemic environment after spinal cord injury leads to limited neuron survival and has complicated the transplantation of cells designed to promote a permissive environment at the implant site [15,16]. The delivery of exogenous VEGF can spare spinal cord tissue, reduce retrograde degeneration, increase blood vessel density, and reduce the number of apoptotic cells [12]. Transplantation of neural stem cells after spinal cord contusion that were genetically modified to produce VEGF enhanced stem cell differentiation into mature oligodendrocytes and improved endogenous gliogenesis, angiogenesis, and tissue sparing [17]. However, a significant challenge is to control the release of these factors, as there is the potential for increased microvascular permeability, associated with leukocyte infiltration and an increased lesion volume. In addition to enhancing vessel ingrowth, angiogenic factors may be able to promote axonal ingrowth. Both the vascular and neural networks are structurally similar complex branched systems that are wired by multiple regulatory factors [18,19]. Several axon guidance cues are involved in blood vessel guidance [19,20], while VEGF and FGF-2 have been suggested to play a role in nerve regeneration [2,4,21]. VEGF can pattern small arteries along peripheral nerves [22], stimulate neurogenesis of neural stem cells in the adult central nervous system [23], have a mitogenic effect on neural cell types, such as astrocytes [24] and Schwann cells [25,26], and induce limited regeneration of the corticospinal tract across the injury site [13]. Although the delivery of angiogenic factors has many potential advantages after spinal cord injury, their controlled release from bridges has, to the best of our knowledge, not yet been investigated.

In this report, we implanted poly(lactide-co-glycolide) (PLG) bridges with the ability to locally deliver VEGF and FGF-2 to a spinal cord injury and analyzed blood vessel formation, endothelial cell infiltration, and nerve growth. Bridges were fabricated using a gas foaming/particulate leaching process and have previously demonstrated good tissue apposition without cavity formation, cell ingrowth into the pores of the bridge, and the ingrowth of neurofilament-positive fibers [6,27], which contrasts with the insertion of Gelfoam that resulted in cavity formation. VEGF and FGF-2 were loaded into the bridge through either encapsulation into the microspheres and/or direct mixing of microspheres with protein before gas foaming. The encapsulation efficiency, release, and bioactivity of the released protein was characterized *in vitro*, while the presence of exogenous VEGF at the injury site *in vivo* was analyzed 1 week post implantation in a rat spinal cord hemisection model. Non-invasive high resolution X-ray micro-computed tomography (micro-CT) was used to visualize the 3D position and formation of functional blood vessels for multiple protein doses *in vivo* [28]. Subsequent studies investigated two VEGF/FGF-2 combinations for the promotion of angiogenesis, and neurotrophic effects, such as axonal extension into the lesion. Angiogenic factor delivery may be employed to combat the ischemic environment that can induce cell death and limits cell transplantation approaches to promote spinal cord regeneration.

Materials and methods

Fabrication of protein loaded multiple channel bridges

Protein loaded multiple channel bridges were made of PLG and fabricated using a combination of a cryogenic double emulsion technique and a gas foaming/particulate leaching technique, which have been previously described [29,30]. The technique in these reports was adapted to the fabrication techniques for multiple channel bridges, which have a more complex architecture based on its application to spinal cord regeneration [5,6,8,27]. A mixture of protein loaded microspheres (2.6 mg), lyophilized proteins, and salt as a porogen (10 mg, 63–106 μm) was mixed with 0.5 μL water using the wet granulation method [31], and deposited in a custom made mold layer-by-layer before gas foaming. This resulted in a porous 3D bridge after 1 h leaching in water, which is sufficient to leach out all the salt, based on the dry weight of the bridges [6]. The bridges contained 7 channels and were approximately 90% porous based on their volume, weight, and the density of PLG [6]. Their dimensions were 4 mm in length, 2.6 mm in width, and 1.5 mm in height, which matched the dimensions of the lateral hemisection created in the rat spinal cord [6,27]. Images of the bridges can be found in these previous reports and a schematic is provided in Fig. 4, A.

Proteins were encapsulated into the microspheres by emulsifying a protein solution (17 μl), containing VEGF (recombinant mouse VEGF-165, Prospec, Rehovot, Israel) or FGF-2 (recombinant human FGF-2, Chemicon, Billerica, Massachusetts), 700 μg bovine serum albumin (BSA), 50 mg/mL sucrose, and MgCO_3 (3% wt of BSA), with 500 μL 3 wt% PLG in dichloromethane using sonication at 40 watt for 15 s on ice. This first emulsion was sequentially frozen in liquid nitrogen for 4 sec in order to freeze the aqueous protein solution part without freezing the polymer solution. In a second emulsion, the first protein/polymer emulsion was added to 25 mL of 5% poly (vinyl alcohol) (PVA, 88% hydrolyzed, average MW 22,000, Acros Organics, Morris Plains, NJ) containing 50 mg/mL sucrose, and homogenized at 7000 rpm for 45 s. The resulting solution was diluted in 15 mL 1% PVA with 50 mg/mL sucrose and stirred at room temperature for 3 h. Microspheres were collected by centrifugation, washed three times, and lyophilized overnight.

For the mixing method, protein was lyophilized with 15 μL 1% alginate and mixed with the microspheres (5 wt% alginate to PLG [32]) and salt before loading into the mold for bridge fabrication. The initial amount of protein added to the protein solutions was based on the encapsulation efficiency and losses during leaching measured by radioactive I_{125} -labeled VEGF and FGF-2.

Protein encapsulation efficiency and release from bridge

The release kinetics for VEGF and FGF-2 from multiple channel bridges was determined using radiolabeled protein. A total of approximately 0.1 μCu I_{125} -labeled VEGF and FGF-2 (Perkin Elmer, Waltham, MA) and 0.2 μg non-radiolabeled protein was either mixed with and/or encapsulated within the microspheres per bridge to quantify the encapsulation efficiency and losses during leaching. The growth factor was mixed with an excess of BSA (100 μg) for encapsulation; thus, small changes in growth factor loading were not expected to substantially alter the bioactivity or release profile [33]. To measure the encapsulation efficiency, a known amount of microspheres was dissolved in 5M NaOH to measure its radioactivity using a Gamma counter (Micromedic 4/600 Plus, Micromedic, Horsham, PA). The efficiency was calculated as the ratio of protein loading per mg microspheres and the maximum protein loading, which was calculated as the ratio of the initial amount of protein added to the PLG solution and the PLG weight in this solution. The losses during leaching were calculated by measuring the leaching fluid. After leaching, the bridges were dried, incubated in 1 mL phosphate buffered saline (PBS) at 37 °C, and transferred to fresh PBS at

specific time points. The activity of the PBS that contained the bridge for that specific time period was measured to quantify the amount of protein that was released. The cumulative release of VEGF and FGF-2 from the bridges was calculated by dividing the cumulative radioactivity released into the buffer by the total amount of radioactivity released over the course of the study plus the radioactivity that remained inside the bridge at the end of the study. The latter was analyzed by dissolving the bridges in 5M NaOH to measure its activity.

Bioactivity of protein released from bridge

The activity of released VEGF and FGF-2 was measured by their ability to phosphorylate the VEGF receptor 2 (R2) and FGF-2 receptor, respectively, of human umbilical vein endothelial cells (pre-screened HUVECs, ECACC, # S200-05n). Bridges containing 1 µg of VEGF or FGF-2 (encapsulated or mixed, n=3) were incubated in 500 µL of PBS at 37 °C. At day 1, 5, 10, 20 and 42, release solutions were collected and replaced by fresh PBS. Due to the low protein amount in release solutions, the release buffer of the triplicates were combined before incubation with HUVECs. HUVECs were first incubated 4 hours in serum-free medium, and then incubated with the release buffer for 5 minutes. Cells were washed with cold HBSS and lysed for 15 minutes in 50 µL of ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, 0.05 % sodium deoxycholate, 1 mM EDTA, 0.1 % SDS and protease inhibitors (Protease Inhibitor Cocktail, Sigma)). Cell debris was removed by centrifugation at 14,000 × g for 10 minutes at 4°C and protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). Cell extracts (70 µL) were then resolved by 6% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). VEGF and FGF-2 that were not encapsulated within the bridge were used as positive controls.

Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk powder in 1 M Tris (pH 7.4), 5 m NaCl, and 0.05% Tween 20 (TBST), and incubated overnight at 4°C with the primary antibody rabbit anti-phospho-VEGFR2 (TYR1175) or rabbit anti-phospho-FGF R (TYR653) (Cell Signaling Technology, Beverly, MA) diluted 1/1000 in 1% nonfat dry milk powder in TBST. After washing in TBST (3 × 15 minutes), membranes were incubated for 1 hour at room temperature in peroxidase-conjugated anti-rabbit secondary antibody (0.1 µg/ml, Jackson ImmunoResearch Laboratories, Suffolk, UK). After washing in TBST, enhanced chemiluminescence was done according to the manufacturer's recommendation (Immobilon™ Western, Millipore). Protein expression was analyzed via Geliance 600 (Perkin Elmer).

Rat spinal cord hemisection model

To analyze the performance of the bridges *in vivo*, bridges were implanted into a rat spinal cord hemisection model [5,6,27]. Thirty four female Long-Evans rats (Charles River, 180–200 g) were treated according to IACUC guidelines at Northwestern University, and pre-handled for two weeks pre-surgery. Rats were anaesthetized using a RC2 Rodent Anesthesia System (Colonial Medical Supply, Franconia, NH) with vaporized Isoflurane (Baxter, Deerfield, IL) to perform a laminectomy at T9–10 and expose the spinal cord. A lateral hemisection of 4 mm long up to the midline was created for bridge implantation, after which the injury site was covered with Gelfoam (Henry Schein, Melville, NY), the muscles were sutured together, and the skin stapled. Post-operative care included administering Baytril (Enrofloxacin 2.5 mg/kg s.c., once a day for 2 weeks), buprenorphine (0.01 mg/kg s.c., twice a day for 2 days), and lactate ringer solution (5 mL/100 g, once a day for 5 days), and bladder expression twice a day until bladder function recovered.

ELISA to quantify VEGF at the injury site

An enzyme-linked immunosorbent assay (ELISA) was performed to analyze the presence of delivered VEGF at the injury site and its adjacent spinal cord segments 1 week post injury. The bridges (n=4) were fabricated with 0.5 µg rat VEGF (kindly provided by Prof. Carmeliet, Katholieke Universiteit Leuven, Belgium) encapsulated inside the microspheres plus 0.5 µg rat VEGF mixed with the microspheres. This loading of VEGF was sufficient to determine if VEGF delivery was increasing levels above background. Control implants (n=4) were done with bridges without VEGF. Upon removal of the spinal cord, the Gelfoam was removed from the cord and the cord was cut into 3 segments: the injury site and two 0.5 cm long segments rostral and caudal of the injury site (Fig. 4, A). All segments were stored on dry ice until being thawed and cut into small pieces. Lysis buffer (100 µL) (Cell Culture Lysis Reagent 1X, Promega, Madison WI) was added, and the lysate was vortexed for 15 sec, rotated for 30 min using a rotamix (Appropriate Technical Resources, Inc., Laurel MD), and centrifuged at 14,000 RPM for 10 min at 4°C to collect the supernatant. The supernatant was diluted (1/200) to perform the VEGF ELISA, according to supplier instructions (Rat VEGF ELISA kit for cell and tissue lysate, RayBio®, RayBiotech, Inc, Norcross GA). Note that this processing of the spinal cord does not solubilize the polymer; thus, the procedure measures the protein within the tissue and not within the polymer.

Micro-computed tomography to analyze functional blood vessels

MicroCT was performed as an initial qualitative screening for 5 different VEGF/FGF-2 doses ranging from 1 to 2 µg of one or both proteins (n=3) to visualize the presence of functional blood vessels in the bridge after spinal cord injury and determine the doses for a more quantitative analysis of cell infiltration [34–36]. To analyze this functional response of protein delivery *in vivo*, greater protein amounts were used compared to the release studies. Fourteen rats (1 rat died) were euthanized at 6 weeks post implantation using a transcardiac perfusion to inject Microfil compounds (Flow Tech, Inc., Carver, MA) that cure to form a three-dimensional cast of the animal's vasculature, which can then be imaged by microCT. Animals were deeply anesthetized using Euthasol to open the abdominal cavity, cut the diaphragm, and expose the heart. A perfusion needle was inserted into the left ventricle oriented to the ascending aorta, while the right atrium was opened using surgical microscissors. Animals were perfused at 20 mL per minute with phosphate buffered saline (PBS) (50 mL), followed by 4% phosphate buffered paraformaldehyde (300–400 mL) to fix the tissue, and 50 mL of PBS to rinse out paraformaldehyde.

The Microfil injection compound (MV-122) was prepared by adding 500 µL of curing agent per 10 mL MV-122 and thorough mixing. It was sequentially manually injected at a slow rate into the animal's vasculature, using a 10 mL syringe and a 21-gauge needle. This procedure fills blood vessels that connect to the animal vasculature, and are referred to as functional vessels. After curing, the spinal cords were removed for image analysis with a high-resolution *in vitro* micro-CT system, SkyScan 1072 (SkyScan, Kontich, Belgium). The scanner was equipped with a point X-ray source (8 micron focal spot) operating at 80kV and 100µA and the study was performed with 10 micron pixel size without filter. These conditions allow imaging the smallest vessels with optimal contrast provided by the soft part of X-ray spectra. From the virtual cross-sections, 3D models were created using ANT software (Skyscan, Kontich, Belgium) in order to visualize the position of the blood vessels.

Immunohistochemistry

The effect of VEGF and FGF-2 delivery from the bridges on the presence of angiogenic markers, such as endothelial cells, and neurotrophic effects, such as nerve extension into the lesion, was sequentially investigated using immunohistochemistry for three different conditions: bridges containing 4 µg of VEGF encapsulated within their microspheres and 2

μg of FGF-2 and VEGF mixed (high protein dose, n=4), 4 μg of VEGF encapsulated within their microspheres and 1 μg of FGF-2 and VEGF mixed (medium protein dose, n=3), and bridges without protein loading (no protein, n=4).

Rats (n=11) were sacrificed at 6 weeks post implantation to retrieve the spinal cords, and prepared for immunohistochemistry as described previously [27]. The tissue section containing the injury site (T8–T11) was frozen in isopentane (Fisher Scientific, Pittsburgh, PA), stored in siliconized eppendorf tubes at -80°C , cryopreserved in optimum cutting temperature (O.C.T.) compound, and sliced longitudinally in 10 μm thick sections using a cryostat (Micron, Microm HM 505 N). Every other section was collected on poly (L-lysine) coated glass slides (Fisher Scientific), post-fixed, and stained.

Each 10th collected section was stained for a specific cell stain with primary IgG1 antibodies against endothelial cells (mouse-anti-rat RECA-1, AbD Serotec, Raleigh, NC, 1:150 dilution) and neurofilament (rabbit-anti-NF 200; Sigma-Aldrich; 1:5000 dilution) in combination with a secondary immunoperoxidase stain (biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, 1:200 dilution) and anti-rabbit IgG (Jackson Lab, West Grove, PA, 1:200 dilution), respectively). Negative controls were performed by eliminating the primary antibodies. Images were taken of the entire bridge section in phase (20X) and 6 sections per rat were analyzed for each stain in Photoshop. The results were reported as a percentage of the surface area of the entire bridge that was stained positive, and normalized to the results obtained for bridges without protein. The surface areas of the positive stain and the entire bridge were measured by selecting these areas and measuring their total amount of pixels using the histogram tool in Photoshop.

Statistical analysis

Statistical analyses were done using statistical package JMP (SAS, Cary, NC). For multiple pairs comparison, an ANOVA with post-hoc Tukey test was performed with a p-level of 0.05. A t-test was performed to analyze differences between individual pairs. Error bars represent standard deviations in all figures.

Results

VEGF and FGF-2 encapsulation and release from bridges

The encapsulation efficiency of the proteins within the bridge, losses during leaching, and release profile either by encapsulation within microspheres or mixing with microspheres was initially investigated. High molecular weight 75:25 PLG was used to encapsulate VEGF and FGF-2 into PLG microspheres, resulting in encapsulation efficiencies of 44 ± 16 and $56 \pm 14\%$, respectively, which were not statistically different (Fig. 1, A). Varying the polymer solvent agent (ethylacetate and dichloromethane), the lactide to glycolide ratio (85:15, 75:25, 65:35 PLG), or the PLG concentration (3, 5 %) did not enhance the encapsulation efficiency (data not shown). Protein incorporated inside the bridges through initial encapsulation into microspheres resulted in minimal protein losses during the leaching process, with protein retention of $93 \pm 0\%$ and $95 \pm 2\%$, respectively, inside the bridge. Bridges fabricated by mixing the protein with the microspheres prior to gas foaming had a retention of 41 ± 5 and $52 \pm 1\%$, respectively, after leaching (Fig. 1, B), which was statistically different for both proteins. Importantly, the total amount of protein that can be loaded inside the microspheres is limited due to the first emulsion volume and the initial protein concentration, which is not a limiting factor in the case of protein mixing with the microspheres.

The release study was subsequently investigated, and release was a strong function of the incorporation method and the protein incorporated. All conditions had an initial rapid release

that persisted for 4 days, followed by a more gradual release. Bridges loaded with VEGF demonstrated a faster release compared to FGF-2 for both incorporation methods with a greater percentage released after 23 weeks. For bridges with mixed VEGF or FGF-2, respectively, the initial phase led to a release of $61 \pm 9\%$ and $27 \pm 6\%$, while the second phase released $26 \pm 6\%$ and $36 \pm 3\%$ during the subsequent 157 days. For encapsulated VEGF or FGF-2, respectively, the initial phase had a release of $21 \pm 4\%$ and $12 \pm 1\%$, and a sustained release during the subsequent 157 days with a maximal release of $57 \pm 4\%$ and $40 \pm 2\%$ of the encapsulated protein. Bridges that were loaded with protein by both mixing and encapsulation inside the microspheres had an intermediate release profile (Fig. 2).

Bioactivity of protein released from bridge

The bioactivity of VEGF and FGF-2 was subsequently determined for protein that was released from bridges in which the protein was encapsulated within the microspheres, or mixed with the microspheres. Phosphorylation of VEGF-R2 and FGF-R was observed for protein obtained following release for 24 hours, 5, 10, 20, and 42 days (Fig. 3), with activity observed for protein that was mixed with or encapsulated into the microspheres. Variations of intensity between samples are due to the variability in protein content for the release solutions. This activity of VEGF and FGF-2 was consistent with multiple reports involving the release of these proteins from PLG [35,37].

VEGF localization at the injury site

VEGF loaded bridges were subsequently implanted into the lateral hemisection. We have previously reported that these bridges prevent the formation of a cyst-like cavity typical of many spinal cord injuries, likely due to stabilizing the injury following implantation [6,27]. Cells infiltrate into the pores and channels of the bridge within a week, with the channels aligning cells along the major axis of the channel. The presence of the delivered protein at the injury site was analyzed by quantifying the amount of protein in the injury site and in adjacent spinal cord segments (Fig. 4, A). Bridges were loaded with 0.5 μg VEGF inside microspheres and 0.5 μg VEGF mixed with microspheres, a loading that was sufficient for quantification by ELISA. VEGF levels at the injury site were 20-fold greater than for bridges without VEGF (Fig. 4, B). Additionally, for VEGF loaded bridges, VEGF levels at the injury site ($4300 \pm 1924 \text{ pg/mL}$) were significantly greater than the levels in the gelfoam covering the implant or in the segments rostral and caudal to the injury site. These results suggest that the delivered protein remained local at the injury site 1 week post implantation, with VEGF levels significantly greater than the endogenous VEGF levels after spinal cord injury.

Functional blood vessels formed following protein delivery

The presence of functional blood vessels in the bridge after implantation to the spinal cord hemisection was characterized by microCT. As a control, the vascular network of an uninjured spinal cord was filled with contrast agent to validate the technique (Fig. 5, A). The protein loadings that were employed were selected from literature reports in which PLG scaffolds were used to deliver VEGF *in vivo* [34–36]. The combinatorial delivery of VEGF and FGF-2 was investigated based on the potential for synergistic activity between the two factors to promote blood vessel development [38–41]. Following bridge implantation for 6 weeks, a greater extent of blood vessels was visualized inside bridges when a high dose of VEGF (2 μg) was encapsulated within the microspheres and when FGF-2 (1 μg) was mixed with the microspheres (Fig. 5, B). Bridges with an intermediate VEGF dose (1 μg), or without the proteins did not demonstrate the same extent of blood vessel growth after spinal cord injury. Mixing FGF-2 with the microspheres in addition to VEGF encapsulation (1 μg) enhanced blood vessel ingrowth compared to VEGF mixed with the microspheres (Fig. 5,

B), while additional encapsulation of FGF-2 in the microspheres did not enhance blood vessel growth (data not shown).

Infiltration of endothelial cells in bridge

The presence of endothelial cells in the bridge was subsequently evaluated at 6 weeks post implantation by immunohistochemistry. Based on the qualitative microCT results, which demonstrated that bridges with intermediate doses of protein did not result in the same extent of blood vessel ingrowth as bridges with the maximal doses, and that bridges with FGF-2 mixed with the microspheres in addition to VEGF enhanced the presence of blood vessels at the injury site, the protein doses were increased to ensure that the protein loading was sufficient to promote endothelial cell infiltration and axonal extension into the bridge. The dose increase was also done to account for the flow of cerebrospinal fluid that may enable convective transport of the proteins from the implant [5]. Therefore, one dose of VEGF (4 µg) was encapsulated in microspheres and 2 different doses of VEGF and FGF-2 were mixed within the bridge.

For VEGF and FGF-2 releasing bridges, substantial RECA staining was observed within the bridge and adjacent tissue, (Fig. 6, A), while intact circular shaped blood vessels were detected adjacent to the bridge (Fig. 6, B). Bridges loaded with medium or high protein dosages had significantly greater RECA staining relative to bridges without protein (Fig 6, C). Bridges loaded with 4 µg of VEGF encapsulated within their microspheres and 2 µg of FGF-2 and VEGF mixed (high protein), had a 3.3 ± 1.0 fold greater RECA staining relative to bridges without protein. Bridges containing 4 µg of VEGF encapsulated within their microspheres and 1 µg of FGF-2 (medium protein) had a 2.5 ± 0.7 fold greater RECA staining. Differences between bridges with medium and high protein doses were not statistically significant.

Neurite growth into the bridge

The extent of neurite growth within the bridge at 6 weeks post implantation was subsequently characterized in order to investigate synergy between the endothelial cells and neurite outgrowth. Substantial neurofilament (NF) staining was present in all conditions (Fig. 7, A). For bridges without protein or with the intermediate levels of VEGF/FGF-2, the mean level of staining was similar (Fig. 7, B). For bridges loaded with the highest dose of VEGF and FGF-2, the mean staining level was greater (1.7-fold relative to no protein, Fig. 7, B); however, this difference was not statistically significant.

Discussion

In this report, VEGF and FGF-2 were delivered to the injured spinal cord from a bridge to enhance blood vessel formation and reduce ischemia, and to investigate if the delivery of these factors impacts neurite outgrowth. Angiogenic factors, such as VEGF and FGF-2 have been delivered from PLG scaffolds formed by the gas foaming process in order to increase blood vessel formation locally [29,32,42–44], yet these strategies have not been applied to the spinal cord. The gas foaming process has been effective in previous reports at maintaining the bioactivity of both VEGF and FGF-2 [29,42,45]. Proteins were loaded into the bridge through i) encapsulation into the microspheres and/or ii) direct mixing with the microspheres, which were subsequently processed by gas foaming to create the bridge structure. The method of protein incorporation impacts the release profile [29], with encapsulation within the microspheres reducing the release rate relative to the mixing procedure. FGF-2 demonstrated a greater encapsulation efficiency and decreased release relative to VEGF. FGF-2 (17.2 kDa) has a smaller molecular weight than VEGF (38.2 kDa), yet has a greater isoelectric point that would impart a more positive charge than VEGF and

could enhance electrostatic interactions with the polymer. Both proteins retained their bioactivity after their release from the bridge up to 42 days.

Delivery of angiogenic factors from a bridge was hypothesized to provide the combination of structural support and biochemical factors to promote revascularization of the injury. In the normal spinal cord, VEGF mRNA is not detectable; however, following spinal cord injury, VEGF expression is upregulated after 6 hrs with a peak at 24 hours [46]. Expression is restricted to the border of the injury, with new vessels present 24 h post injury that eventually disappears with cavity formation and glial scar deposition [46]. These previous studies suggested that delivery should maintain VEGF at the injury and avoid release to the uninjured tissue, and that limiting cavity formation may be essential for long-term vessel stability. Bridge implantation provides mechanical support to the injury and supports cell infiltration, whose combination prevents cavity formation and decreases the extent of glial scar formation [27]. Delivery of 1 µg of VEGF increased VEGF levels 20-fold at the injury site 1 week post implantation relative to bridges without VEGF. The delivered VEGF remained at the injury, as segments rostral and caudal to the injury and the gelfoam had VEGF levels comparable to levels observed with bridges without VEGF. With a VEGF half-life of 3 min in the circulation [47] and 6 min when bound to heparin [48], a 1 week time point suggests a local and sustained delivery of exogenous VEGF from the bridge *in vivo* after implantation, which may have the potential to support the formation of mature blood vessels at the injury site, while avoiding blood vessel retraction.

Localized delivery of angiogenic factors increased endothelial cell infiltration and also the presence of vessels in the adjacent tissue. Significant differences in endothelial cell infiltration into the bridges at 6 weeks post spinal cord injury were observed for bridges releasing VEGF and FGF-2 relative to bridges without proteins. Interestingly, endothelial cell infiltration was not significantly different between medium and high protein doses. This result suggests that a maximal dose was exceeded, or that the mixing process was ineffective at greater doses due to the rapid release. MicroCT analysis indicated the presence and position of functional vessels within the injury site. Furthermore, histological analysis indicated the presence of circular shaped blood vessels adjacent to the bridge in quantities that were increased for bridges containing VEGF and FGF-2 protein relative to no protein. Inside the bridges, however, few circular shaped blood vessels were detected, which may suggest that the pore structure and size might need to be re-evaluated to support blood vessel ingrowth [49,50]. In addition, the relationship between VEGF dosage and the morphology and function of newly formed blood vessels has previously been investigated, demonstrating that elevated VEGF levels can lead to abnormal blood vessels and hemangiomas [51,52]. The studies reported here indicate the potential for localized VEGF delivery to promote angiogenesis locally in the spinal cord. A key challenge will be forming normal mature blood vessels that will restore the integrity of the blood brain barrier. To obtain more mature vessels, multiple research groups are currently investigating a combination of multiple angiogenic factors (e.g., PDGF [53]), their doses, and the time frame of delivery. More research is necessary to overcome the challenges of controlling the time frame and doses of protein release, as parameters may have to be re-optimized for each protein or protein combination.

VEGF has been proposed as a neurotrophin [54], while, more generally, delivery of angiogenic factors may lead to the simultaneous regeneration of blood vessels and nerves. Blood vessels and nerves may be congruent in order to provide nerves with oxygen and nutrients and allow vasoregulation of blood vessels [19]. Additionally, the blood vessels and nerve bundles may provide mechanical support for each other and thus orienting regeneration. In this report, substantial staining for neurofilament was observed in empty bridges (i.e., no protein release), for which there was limited staining for endothelial cells,

suggesting that there is endogenous neurotrophin production that is promoting neurite growth. Delivery of VEGF and FGF-2 increased the mean quantity of staining for neurofilament in the bridge; however, this increase was not statistically significant. Additionally, preliminary staining for astrocytes revealed that the delivery of VEGF and FGF-2 induced astrocytes infiltration into the pores and channels of the bridge (data not shown), which is not observed in the absence of this delivery. Without protein delivery, a dense layer of astrocytes is observed at the edge of the bridge without infiltration inside the bridge, consistent with previous reports [27]. Thus the delivery of angiogenic factors is impacting the local environment and stimulating cells other than endothelial cells.

The significant increase in endothelial cell staining, yet insignificant change in neurofilament staining, with protein delivery may reflect that the pore structure of the scaffold or the quantity or duration of delivery may need to be altered. Although endothelial cell staining was increased within the bridge, the presence of organized vessel structures may be necessary to provide the mechanical support and orientation to promote complementary nerve growth. Altering the pore size has the potential to enhance the number of vessels within the bridge [49,50]. Importantly, the action of VEGF as a neurotrophin is proposed to require long-term VEGF production [13], and the concentration at which VEGF is neurotrophic may differ from the concentration at which it is angiogenic.

Conclusion

Bridges releasing angiogenic factors (VEGF, FGF-2) locally after spinal cord injury increased endothelial cell infiltration and blood vessel formation at the injury, while also supporting neurite outgrowth, which are both necessary to overcome ischemia and promote functional spinal cord regeneration. VEGF and FGF-2 were delivered from the bridge in a controlled manner using a combination of 2 methods of protein loading: encapsulation inside the microspheres and mixing with the microspheres before gas foaming. Bridges stabilized the injury to prevent cavity formation and localized release promoted a local increase of VEGF at the injury, increased endothelial cell infiltration, and enhanced the number of circular shaped blood vessels. This report demonstrates that implantation of bridges releasing proteins into the spinal cord can stabilize the injury while promoting and supporting processes critical to regeneration.

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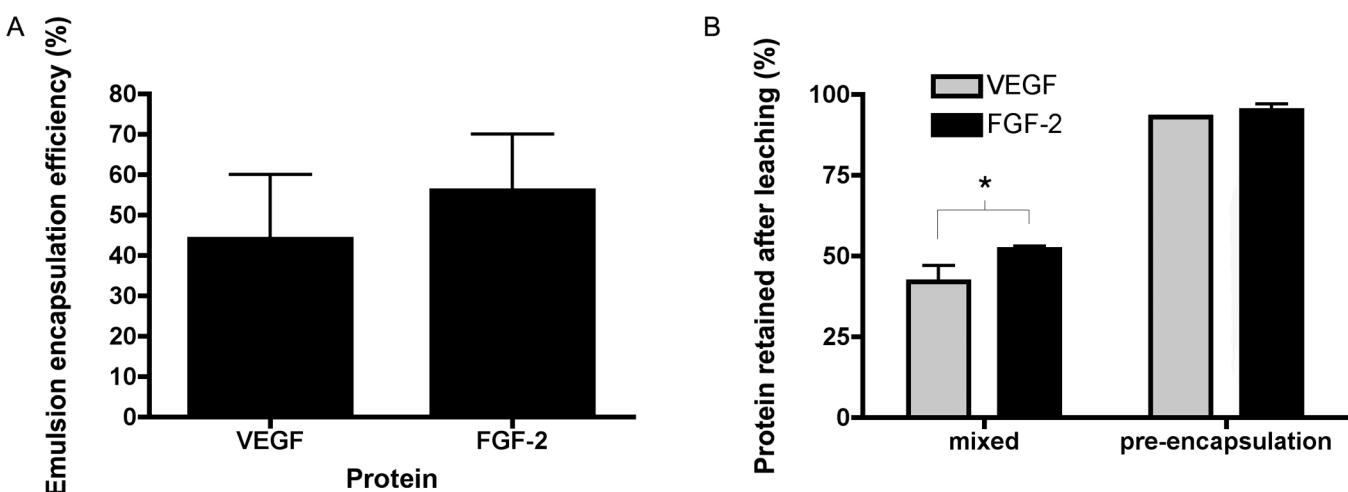
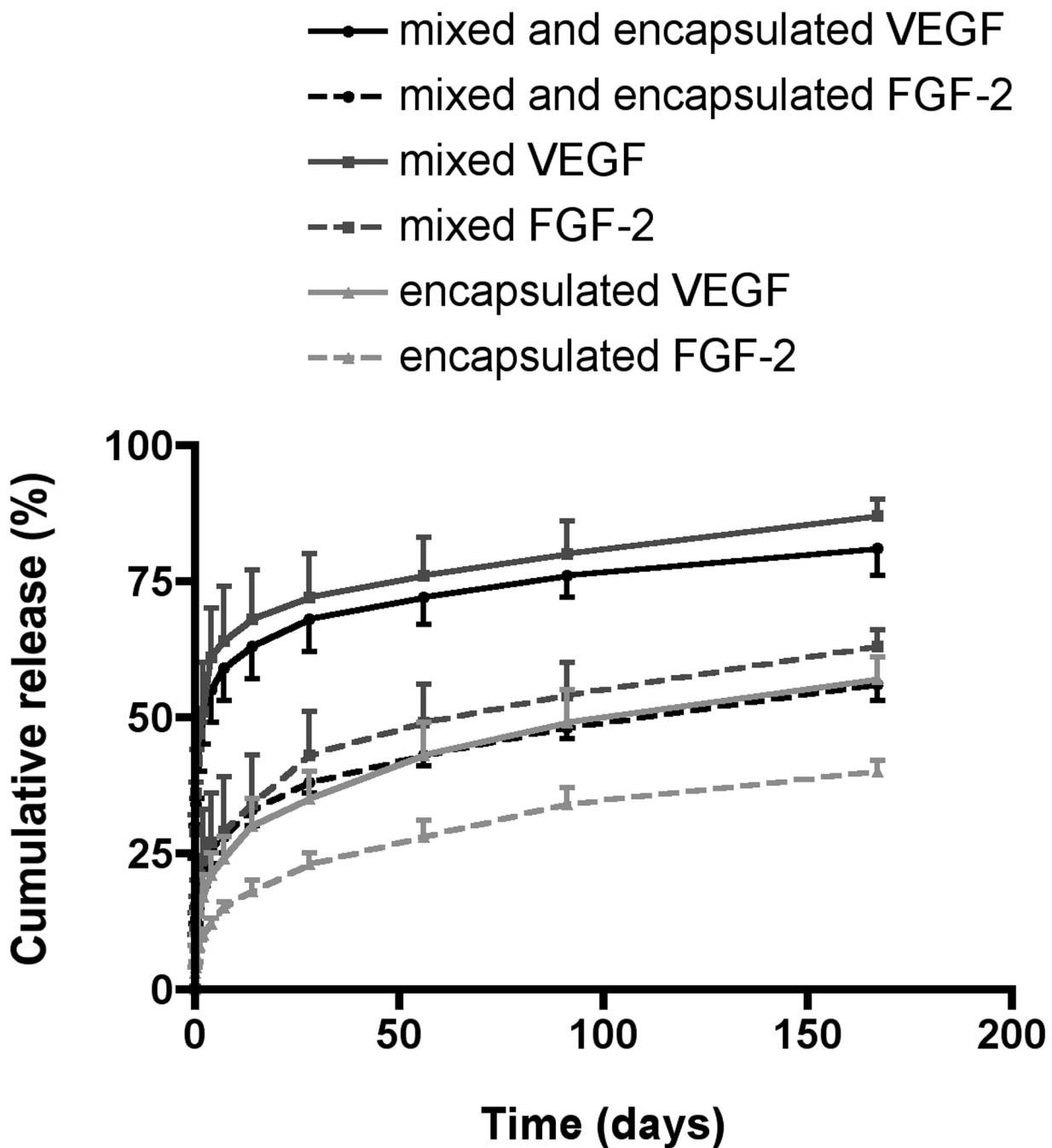
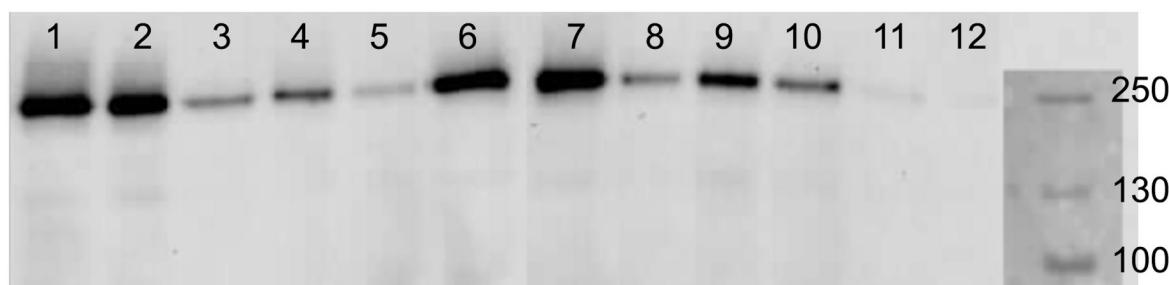
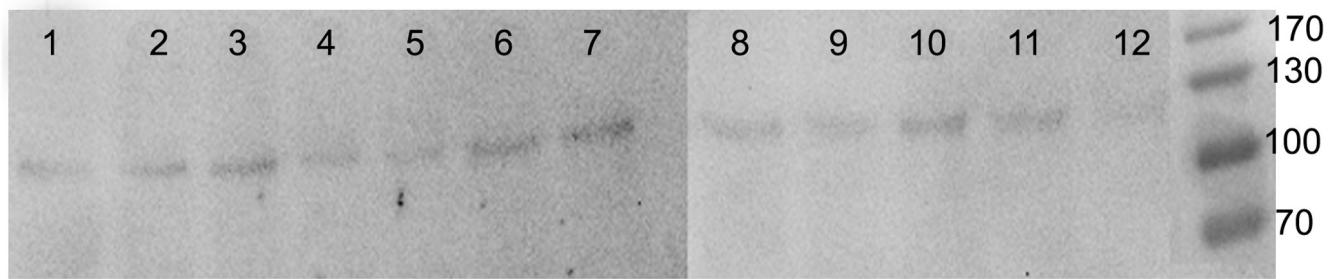


Figure 1.

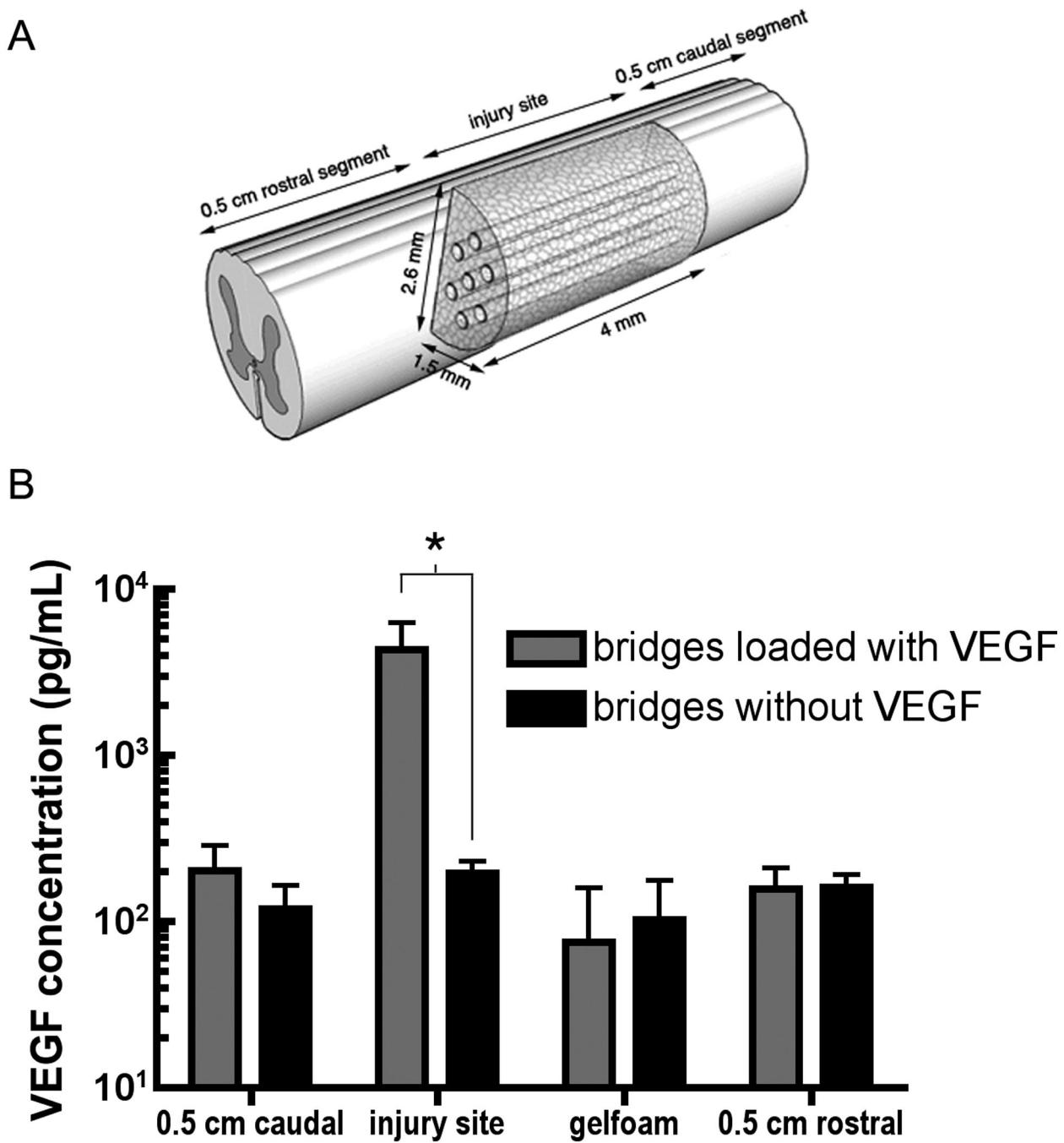
Protein encapsulation efficiency and protein retention after leaching. A) VEGF and FGF-2 encapsulation efficiency after microsphere encapsulation and collection. B) VEGF and FGF-2 retention after leaching bridges for 1 h. Proteins were incorporated using either the encapsulation only or mixing only approaches. Significant differences between protein conditions are denoted by an asterisk ($p<0.05$).

**Figure 2.**

Protein release from bridge. Bridges were fabricated with VEGF (filled line) or FGF-2 (dotted line) in three different manners: mixing only (squares), encapsulation only (triangles), or a combination of both encapsulation and mixing (circles). The amount of protein left in the bridge after leaching was set as 100%.

A VEGF**B FGF-2****Figure 3.**

VEGF and FGF-2 activity during *in vitro* release. Phosphorylation of (A) VEGF and (B) FGF-2 receptors was assessed by western blot. Legend: 1. control (100 ng/ml VEGF or 50 ng/ml FGF-2), 2. mixing 24 hours, 3. encapsulation 24 hours, 4. mixing 5 days, 5. encapsulation 5 days, 6. mixing 10 days, 7. encapsulation 10 days, 8. mixing 20 days, 9. encapsulation 20 days, 10. mixing 42 days, 11. encapsulation 42 days, 12. negative control (PBS).

**Figure 4.**

ELISA for VEGF at the injury and in adjacent tissue at 1 week post implantation. A) Schematic of the spinal cord injury and segments that were analyzed for ELISA, adapted from De Laporte L, Yang Y, Zelivysanskaya ML, Cummings BJ, Anderson AJ, Shea LD. Plasmid releasing multiple channel bridges for transgene expression after spinal cord injury. Originally published in Molecular Therapy 2009;17(2):318–26 [6], with permission of the Nature Publishing Group. B) VEGF levels in different segments for bridges loaded with and without VEGF. Significant differences between conditions are denoted by an asterisk ($p<0.05$).

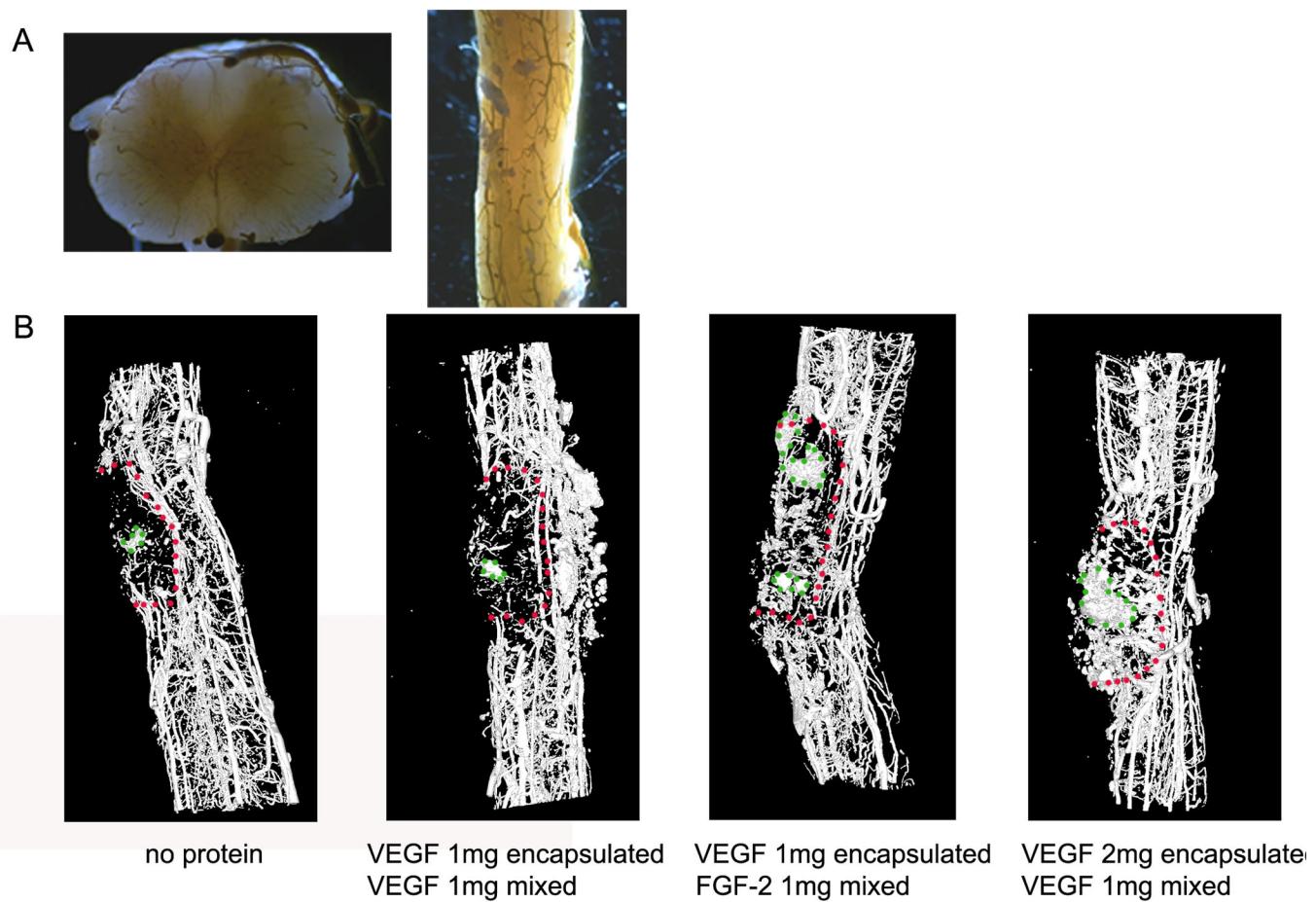


Figure 5.

A) Cured Microfil injection compound that formed a three-dimensional cast of the rat's spinal cord vasculature. From left to right: cross section and longitudinal view. B) 3D reconstructions of micro-CT scans of bridge implants in spinal cord hemisection model at 6 weeks post implantation. From left to right: bridge implant without protein loading, bridge implant with 1 µg VEGF encapsulated and 1 µg VEGF mixed, bridge implant with 1 µg VEGF encapsulated and 1 µg FGF-2 mixed, bridge implant with 2 µg VEGF encapsulated and 1 µg VEGF mixed. The red dotted line marks the contours of the bridge at the implant site. The green dotted line marks the contour of some residual Gelfoam that appeared on microCT. The Gelfoam was used to cover the injury site, and mostly removed upon tissue retrieval.

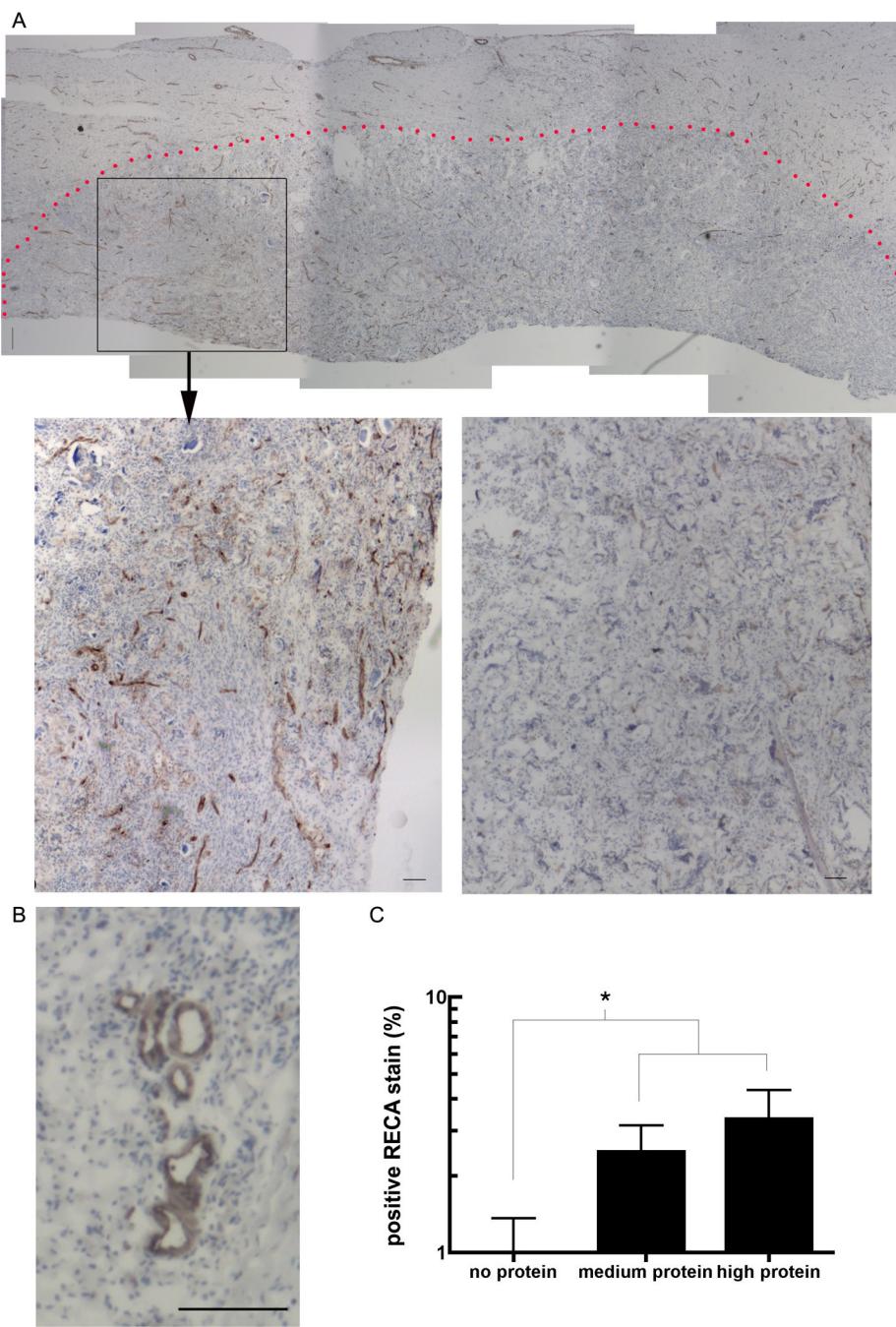


Figure 6.

Endothelial cell infiltration in bridge at 6 weeks post implantation. A) RECA stain (brown): from left to right: bridge containing 4 µg of VEGF encapsulated within microspheres and 2 µg of FGF-2 and VEGF mixed (high protein), and bridge without protein. The red dotted line marks the contours of the bridge at the implant site. Scale bar: 200 µm. B) Blood vessels adjacent to the bridge containing 4 µg of VEGF encapsulated within microspheres and 1 µg of FGF-2 and VEGF mixed (medium protein). Scale bar: 200 µm. C) Quantification of RECA staining for the three conditions.

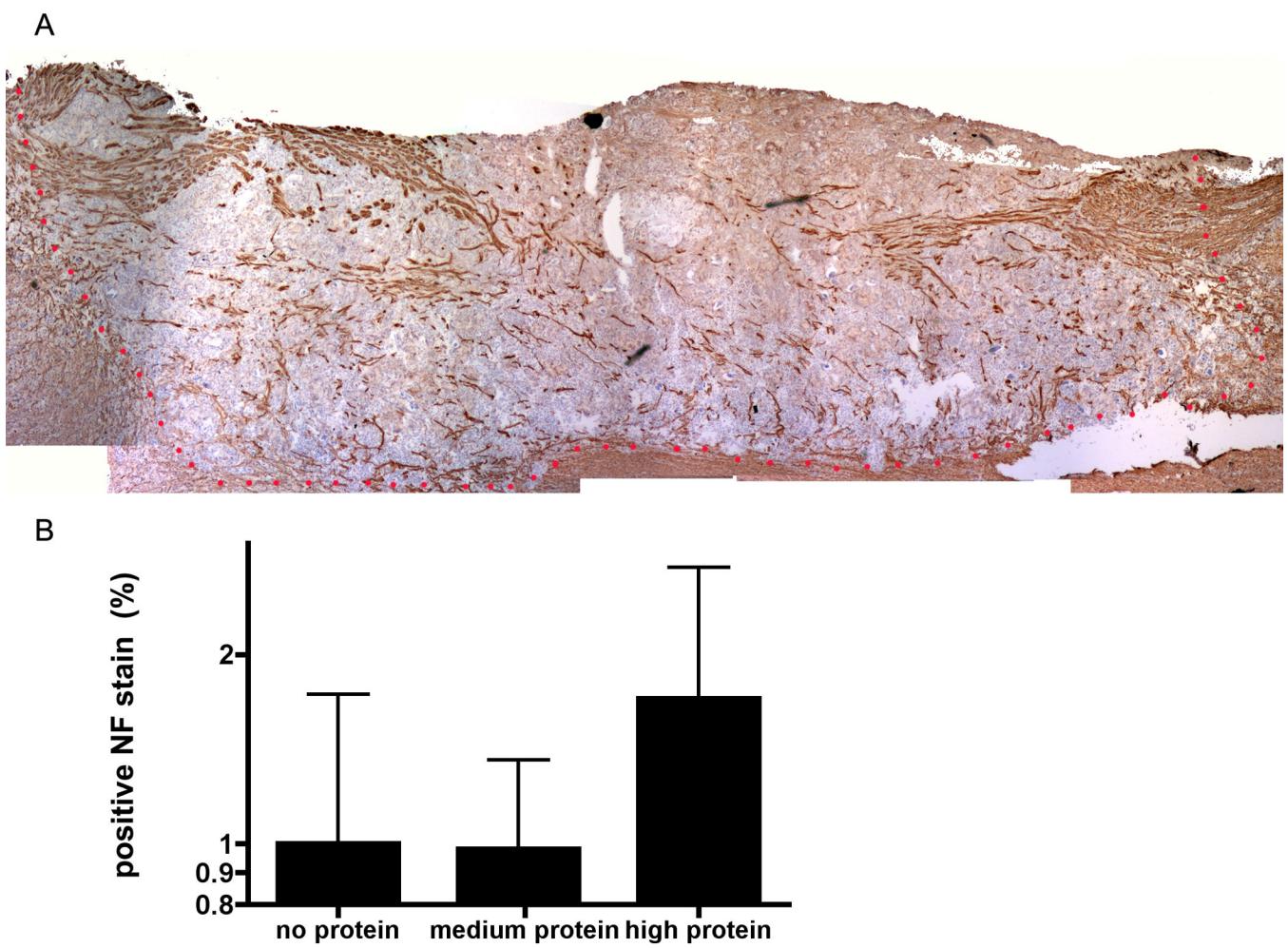


Figure 7.
Neurite ingrowth with bridge at 6 weeks post implantation. A) Neurofilament stain (brown) of bridge implanted with the highest VEGF/FGF-2 protein dose. The red dotted line marks the contours of the bridge at the implant site. Scale bar: 500 μm . B) Quantification of positive NF stain for bridge implants without VEGF/FGF-2, and bridge implants with a medium and high dose of VEGF/FGF-2 protein.