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Dual-delivery of VEGF and NGF by emulsion electrospun nanofibrous scaffold for peripheral nerve regeneration

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ABSTRACT

Controlled delivery of multiple therapeutic agents can be considered an effective approach in nerve injury due to its multifunction. In this study, recombinant human vascular endothelial growth factor (VEGF) and recombinant human nerve growth factor (NGF) were loaded on the surface and in the core of emulsion electrospun poly (L-lactic acid) (PLLA) nanofibrous scaffold, respectively. The *in vitro* studies showed that VEGF and NGF had a sequential release pattern in which most of the VEFG was released in the first few days but the NGF could be continuously released for > 1 month. The dual-delivery scaffold could enhance the neural differentiation of induced pluripotent stem cells-derived neural crest stem cells (iPSCs-NCSCs) *in vitro*. Furthermore, this scaffold was applied to a critical sized defect in rat sciatic nerve model. Footprint analysis, electrophysiological tests, and histological analysis revealed that a significant improvement of neovascularization as well as nerve healing after 3 months post-operation could be achieved by dual-delivery of VEGF and NGF. Taken together, the present study indicated that VEGF and NGF in emulsion electrospun nanofibrous scaffold had a synergistic effect on regeneration of vascularized nerve tissue.

1. Introduction

Peripheral nerve injury is a common global clinical problem, which may significantly affect the patients' ability to perform activities of daily living and cause an enormous socioeconomic burden [1–3]. Autologous nerve grafting remains the gold standard; however, this sacrifices a healthy nerve, requires more extensive surgery and donor nerves are in finite supply [4]. Tissue engineered nerve grafts have emerged as a potential alternative to autologous nerve grafts [5], in which angiogenesis is an essential component of nerve regrowth. Regeneration of endoneural vasculature can precede the outgrowth of axons from the proximal stump [6,7]. The increased metabolic demands of a regenerating nerve can be met through increased neovascularization. Regenerated blood vessels can more effectively deliver oxygen and nutrients, and remove waste products during nerve regeneration. Moreover, as nerve constructs become larger, mass transport issues will become increasingly important. It is very difficult to maintain viability of transplanted and/or infiltrating cells in tissue engineered nerve grafts [8]. Therefore, it is very important to form enough blood vessels before the nerve regeneration in the process of nerve recovering. However, many studies have focused on the neurogenesis effect of tissue engineered nerve grafts, while ignoring the ability of blood vessel formation (angiogenesis) [9]. This deficiency of the nerve conduit affects the functional recovery of regenerated nerve [10]. The tissue engineered nerve grafts should have both the ability of angiogenesis and neurogenesis.

It is found that growth factors have a very good efficiency on enhancing the angiogenesis and neurogenesis of scaffolds. Among these growth factors, nerve growth factor (NGF) has been widely employed to maintain neuronal differentiation. NGF has mostly been encapsulated or supplemented in culture medium, including various tissue-engineering scaffolds for neural tissue regeneration [11]. Lee et al. [12] loaded NGF in a fibrin matrix for controlled release of the growth factor from the matrix. When this matrix was applied to bridge peripheral nerve defects, the axonal growth of the neural cells was significantly enhanced in a dose-dependent manner [12]. In another study, NGF was physically immobilized on the surface of cover slips to promote the proliferation of pheochromocytoma cells [13]. In addition, vascular endothelial growth factor (VEGF) was originally discovered as a growth factor capable of increasing vascular permeability and endothelial cell proliferation, migration, survival, and the well-known effects on angiogenesis [14]. Moreover, VEGF was able to stimulate Schwann cell

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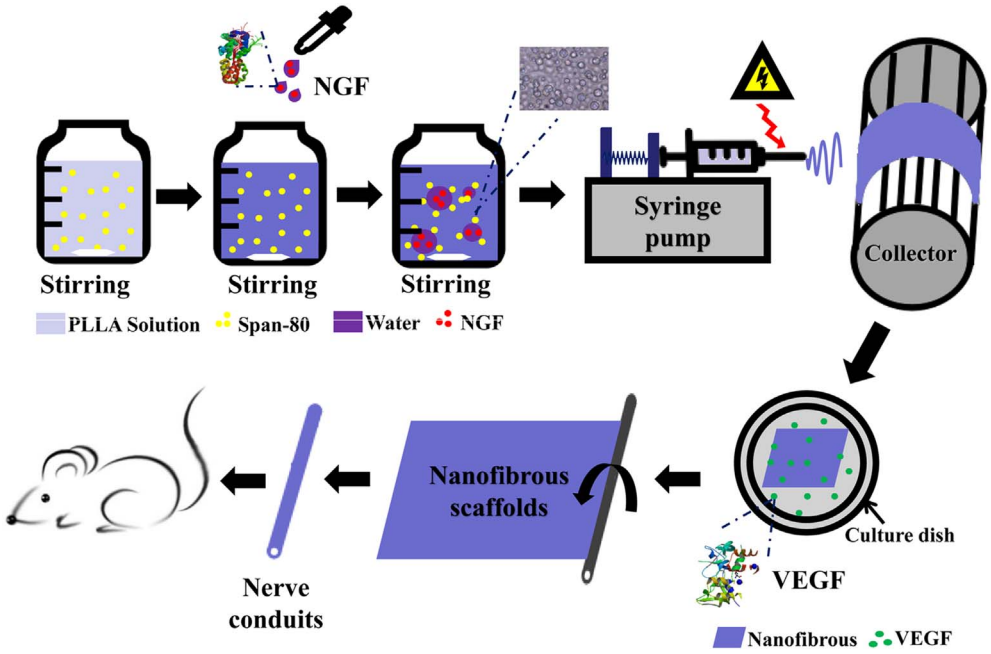

Fig. 1. Schematic illustration of the preparation process of core-shell nanofibrous scaffolds for sciatic nerve regeneration.

invasion and neovascularization [15]. Acellular nerve grafts pretreated with VEGF stimulated the outgrowth of Schwann cells and blood vessels [15]. Du et al. [16] prepared a gradient heparinized nanofibrous graft by electrospinning of chitosan and poly(ε-caprolactone) (PCL) and reported that immobilization of VEGF could enhance the adhesion and proliferation of human umbilical vascular endothelial cells on the lumen of blood vessel and prevent thrombosis.

In addition to selection of growth factors with specific functions, it is recognized that selection of appropriate dual growth factors delivery system is a very important strategy in achieving successful regeneration of vascularized nerve tissue. Layer-by-layer film [17] and core-shell structure scaffold [18] are believed to be the most popular method for loading dual growth factors in tissue engineering applications. Embedding multiple growth factors in charged polyelectrolyte films using layer-by-layer technology can also provide controlled release *via* hydrolytic degradation of films [19]. However, assembly of multilayer films from different materials will be accompanied by many interactions. Besides layer-by-layer, coaxial and emulsion electrospinning have been developed to prepare core-shell fibers for encapsulation of growth factors. The core and shell of the core-shell structure can perform independent functions, such as incorporating two different growth factors [20,21] and preventing decomposition or fast degradation of labile compounds [22]. Compared to the very complex equipment manufacture and electrohydrodynamic behavior of the coaxial electrospinning, emulsion electrospinning is also known as core-shell electrospinning, and is a simple and efficient technology for the fabrication of scaffolds that can provide sustained release of incorporated growth factors [23–25]. The release pattern could be controlled by adjusting the core-shell composition and structure. Besides encapsulating the growth factor in the core of the nanofibrous scaffold, the shell of the nanofibrous scaffold also can adsorb larger amounts of growth factor due to their larger surface areas by the physical adsorption of growth factor on the nanofibrous scaffold [26].

In this work, we developed a new scaffold for effective regeneration with the capability to sequentially release NGF, followed by the release of VEGF in a controlled manner. NGF was initially dispersed in the emulsion turns into the core of the nanofibrous scaffold and then the initial burst of NGF was highly suppressed. Next, the core-shell nanofibrous scaffold was successively functionalized with VEGF *via* physical adsorption. The dual-release profiles of NGF and VEGF were performed, and the cell viability, proliferation, neural differentiation of induced pluripotent stem cells-derived neural crest stem cells (iPSCs-NCSCs) on the nanofibrous membranes were also examined *in vitro*. Furthermore, the regeneration of rat transected sciatic nerve by these nanofibrous nerve conduits *in vivo* was carried out.

2. Materials and methods

2.1. Materials

Poly (L-lactic acid) (PLLA) was purchased from Sigma-Aldrich, Co. (USA). Albumin from bovine serum (BSA) was purchased from Beyotime Institute of Biotechnology (China). β-NGF and $VEGF_{165}$ were purchased from PeproTech Inc. (USA). Dichloromethane and methanol were purchased from Chuandong Chemical Co. (China). CellTiter 96-Aqueous one solution reagent (MTS) was purchased from Promega, Co. (USA). Enzyme-linked immunosorbent assay (ELISA) kits for human β-NGF and human $VEGF_{165}$ were purchased from NeoBioscience Technology, Co. (China). Fluorescein isothiocyanate (FITC) Phalloidin was purchased from Enzo Life Sciences International, Inc. (USA). RNAsimple Total RNA Kit was purchased from TIANGEN Biotech (Beijing) Co. (China). RevertAid first strand cDNA synthesis kit was purchased from Thermo Fisher Scientific Inc. (USA). SYBR Premix Ex Taq was purchased from TaKaRa Biotechnology (Dalian) Co. (China). Rabbit polyclonal anti-neurofilament (NFM) antibody was purchased from Abcam, Inc. (USA).

2.2. Preparation of composite nanofibrous scaffold

Electrospinning of the emulsion solution was performed as described in a previous study with some modifications [21] (Fig. 1). To prepare oil phase, 3 g PLLA was completely dissolved into a mixture solvent containing 3 ml dichloromethane, 0.03 ml span-80 and 0.3 ml methanol and stirred for at least 8 h. To prepare emulsion solution, 0.3 ml water phase was added into 3 ml oil phase drop by drop with vigorous stirring overnight at room temperature till uniform. The water phase was prepared by mixing 0.03 ml of NGF working solution (containing 0.1 μg of NGF) with 0.27 ml of 0.1% BSA aqueous solution. Phosphate-buffered saline (PBS) was used as water phase in the control group.

Before electrospinning, a 7 cm × 34 cm aluminum foil was wrapped around a rotating drum to collect the resultant nanofibrous scaffold.