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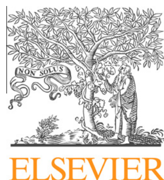

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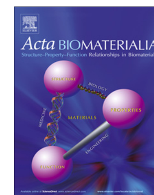

Using NGF heparin-poloxamer thermosensitive hydrogels to enhance the nerve regeneration for spinal cord injury

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ABSTRACT

Objective: Nerve growth factor (NGF) has potential in spinal cord injury (SCI) therapy, but limited by the poor physicochemical stability and low ability to cross the blood spinal cord barrier. Novel heparin-poloxamer (HP) thermo-sensitive hydrogel was constructed to enhance the NGF regeneration on SCI.
Method: NGF–HP thermo-sensitive hydrogel was prepared and related characteristics including gelation temperature, rheological behavior and micromorphology were measured. Local NGF delivery to the injured spinal cord was achieved by *in situ* injection in the injured space. The cellular uptake of NGF–HP hydrogel was evaluated with PC12 cells *in vitro*. Pathologic characteristics and neuron regeneration effects on the SCI rats were studied to evaluate the enhanced therapy of NGF–HP hydrogel. Endoplasmic reticulum (ER) stress-induced apoptosis was analyzed to explore the related mechanism in SCI regeneration.
Results: NGF–HP hydrogel showed good morphology and stable bioactivity of NGF *in vitro*. NGF–HP hydrogel combined treatment significantly enhanced the efficiency of NGF cellular uptake ($P < 0.05$) without obvious cytotoxicity. Significant improvements in both neuron functions and tissue morphology on the SCI rats were observed in NGF–HP hydrogel group. Compared with free HP hydrogel and NGF treatment groups, NGF–HP hydrogel group showed significant inhibition on the formation of glial scars in the extreme crushed rat SCI model. The neuroprotective effects of NGF–HP were related to the inhibition of chronic ER stress-induced apoptosis.
Conclusions: HP hydrogel combined with orthotopic injection technique might be an effective method to deliver NGF into the injured site, which will provide an effective strategy for SCI regeneration.

Statement of significance

Spinal cord injury (SCI) is a devastating condition that can lead to sudden loss of sensory and autonomic function. Current treatment includes decompression surgery, injury stabilization, secondary complications prevention and rehabilitation. However, neurological recovery is limited. Nerve growth factor (NGF) has potential in SCI therapy, but limited by the poor physicochemical stability and low ability to cross the blood spinal cord barrier. Hydrogels have good affinity and compatibility to biological tissue. In this study, we developed a novel heparin-poloxamer (HP) thermo-sensitive hydrogel to enhance the spinal cord regeneration of NGF. From SCI rat experiment, HP hydrogel combined with orthotopic injection technique showed best neuroprotective effects among experimental groups. This novel combined technique will provide an effective strategy for SCI regeneration.
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Abbreviations: SCI, spinal cord injury; NGF, nerve growth factor; ER, endoplasmic reticulum; HP, heparin-poloxamer; NGF–HP, nerve growth factor–heparin poloxamer; BBB, Basso, Beattie, and Bresnahan; BDA, biotinylated dextran amine; GFAP, glial fibrillary acidic protein.

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1. Introduction

Spinal cord injury (SCI) is a devastating condition that is characterized by extensive tissue degeneration and severe loss of function. As one of the most devastating traumas, SCI leads to sudden loss of sensory, motor, and autonomic function distal to the level of injury. Worldwidely, 15–40 new SCI cases occur per million people annually [1]. The primary mechanical injury causes immediate hemorrhage and ischemia, in turn causing hypoxia, inflammation, edema and ultimately widespread cell death. After spinal cord crushed injury, the initial traumatic injury to spinal cord tissues is followed by a long period of secondary damages including oxidative stress, inflammation, necrosis and apoptosis [2,3].

Current treatment includes decompression surgery, injury stabilization, secondary complications prevention and rehabilitation. However, neurological recovery is limited, and substantial neurological dysfunction and lifelong disability still afflicts most SCI patients [4]. Although there is no cure for SCI, advances in molecular medicine based on improved understanding of the pathophysiology of injury have yielded promising results in animal models [5]. These molecules are aimed at either protecting surviving tissue from degeneration or restoring function already lost. The reactive astrocytes are the main obstacle to neuron axon regeneration after SCI. In normal spinal cord, astrocytes can provide material support neuron metabolism, maintain intracellular environment and pH value of the dynamic balance. Astrocytes can also release neurotropic factor to adjust the nerve cells of a variety of functions such as growth, differentiation and metastasis [6–8]. After SCI, astrocytes are activated to become reactive astrocytes [9]. Compared with normal astrocytes, activated reactive astrocytes mainly express in morphology and molecular changes. Activated reactive astrocytes can also express some iconic proteins (such as acid protein, immature collagen glial fiber cells vimentin and nestin) near the injury, and raise some proteins which inhibit the growth of axons expression. All these effects alter the microenvironment of the injury and its surrounding, inhibit the growth of nerve cells and regeneration of axons [10,11].

In the case of SCI, the failure of axonal regeneration is partly resulted from the lack of neurotrophic factors [12,13], in addition to expression of axonal growth-inhibiting molecules and/or inflammatory reactions [14]. In particular, neurotrophic factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have been reported to be beneficial for axonal regeneration when applied to the injury site of the spinal cord [11,15]. NGF is an important member of the neurotrophin family. NGF supports the survival and maintenance of peripheral sensory and autonomic neurons, during development and adult stages [16,17]. Exogenous NGF-administration in developing animals prevents or reduces peripheral neuropathies induced by chemical and surgical insult [18–20]. However, neurons axon regeneration is a slow process. As a macromolecular protein, NGF cannot penetrate the blood spinal cord barrier (BSCB). Therefore, oral and intravenous administration are inaccessible for NGF delivery in SCI. Local epidural or intrathecal delivery by either bolus injection or indwelling catheter/minipump were reported for NGF delivery [21,22].Therefore, novel preparations of NGF are needed to effectively deliver NGF into the spinal cord and maintain sustained release for axon regeneration.

Hydrogels have good affinity and compatibility to biological tissue. With proper formula, hydrogels can load biological macromoleculars to realize controlled release *in situ* [23–25]. In order to overcome the technical bottlenecks in the treatment of SCI, we developed a novel copolymer–heparin-poloxamer (HP) [26], which not only has a good affinity to NGF but also prevents the degradation of protease. In addition, HP has a controlled phase transition according to the variation of temperature. In this study, novel heparin-poloxamer (HP) thermo-sensitive hydrogel was prepared to enhance the spinal cord regeneration of NGF.

2. Materials and methods

All experimental rats were raised in the same environment and randomly selected for each group. The neurobehavioral observation and neuroanatomical evaluation were performed by reviewers who were "blinded" to experimental conditions.

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Nerve growth factor (NGF) was ordered from Gelusite Biology Technology Company, Zhejiang, China. Poloxamer 407–grafted heparin copolymer was synthesized by our laboratory as previously reported [26]. All other chemicals were used as received without further purification. Glial fibrillary acidic protein (GFAP), Caspase-3 and CD31 antibodies were purchased from abcam. Goat anti-rabbit and anti-mouse IgG-HRP were purchased from abcam. All of the other reagents were purchased from Beyotime institute of Biotechnology (shanghai, China) unless otherwise specified.

2.2. Preparation of NGF–HP hydrogels

Heparin-poloxamer (HP) was prepared according to EDC/NHS method as described previously [27]. NGF–HP hydrogels containing different amounts of HP and NGF were prepared using the cold method [2]. In brief, lyophilized HP powder was mixed with NGF solution (phosphate buffered saline, pH = 7.6) at 4 °C under gentle stirring. The mixture was kept in a refrigerator at 4 °C overnight until a clear solution was formed.

2.3. Micromorphology of NGF–HP hydrogel

Gelation temperature measurement and rheological behavior of NGF–HP hydrogels were measured in triplicate and the average value of each point was reported [28]. The micromorphology of the dehydrated NGF–HP hydrogels was observed by scanning electron microscope (SEM). The NGF–HP hydrogels were wiped on a copper sheet, and immersed into liquid nitrogen immediately. Then NGF–HP hydrogels were critical point dried by vacuum freeze dryer for 24 h. The dehydrated specimens were cross-sectioned and sputter-coated with gold, and their surface morphology was observed in a scanning electron microscope (Hitachi, H-7500, Japan) [29,30].

2.4. Cell Viability Assay and axonal growth

PC12 cells were purchased from the Cell Storage Center of Wuhan University (Wuhan, China). PC12 Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 5% horse serum, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin), incubated in a humidified atmosphere containing 5% CO2 at 37 °C. PC12 cells were seeded on 96-well plates(5×10^3 cells/well) for 24 h and treated with different doses of Hydrogen peroxide solution (H_2O_2, 0, 50, 100, 200, 300, 400, 600 μmol/L). From the cells survival state, 400 μmol/L H_2O_2 concentration was selected for the subsequent experiments [Fig. S3].

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