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Electrical regulation of olfactory ensheathing cells using conductive polypyrrole/chitosan polymers

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ABSTRACT

Electrical stimulation (ES) applied to a conductive nerve graft holds the great potential to improve nerve regeneration and functional recovery in the treatment of lengthy nerve defects. A conductive nerve graft can be obtained by a combination of conductive nerve scaffold and olfactory ensheathing cells (OECs), which are known to enhance axonal regeneration and to produce myelin after transplantation. However, when ES is applied through the conductive graft, the impact of ES on OECs has never been investigated. In this study, a biodegradable conductive composite made of conductive polypyrrole (PPy, 2.5%) and biodegradable chitosan (97.5%) was prepared in order to electrically stimulate OECs. The tolerance of OECs to ES was examined by a cell apoptosis assay. The growth of the cells was characterized using DAPI staining and a CCK-8 assay. The mRNA and protein levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neural cell adhesion molecule (N-CAM), vascular endothelial growth factor (VEGF) and neurite outgrowth inhibitor-A (NOGO-A) in OECs were assayed by RT-PCR and Western blotting, and the amount of BDNF, NGF, N-CAM, VEGF and NOGO-A secreted was determined by an ELISA assay. The results showed that the PPy/chitosan membranes supported cell adhesion, spreading, and proliferation with or without ES. Interestingly, ES applied through the PPy/chitosan composite dramatically enhanced the expression and secretion of BDNF, NGF, N-CAM and VEGF, but decreased the expression and secretion of NOGO-A when compared with control cells without ES. These findings highlight the possibility of enhancing nerve regeneration in conductive scaffolds through ES increased neurotrophin secretion in OECs.

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1. Introduction

Lengthy nerve defects need grafting conduits to repair [1]. Although autogenous nerve grafting is still considered the therapeutic gold standard method for bridging nerve defects [2], it has many disadvantages including limited donor grafts availability and postoperative complications of donor sites, such as scarring and neuroma formation [3]. Therefore, bridging lengthy nerve defects without sacrificing healthy nerves to obtain the nerve grafts have significant clinical importance. Driven by this consideration, several alternative types of conduits (biological or synthetic) have been made in the field of neural tissue engineering with an attempt to fabricate nerve scaffolds, which can guide nerve regeneration as alternatives to nerve autografts. To date, most of the studies have been performed mainly on optimizing the inner microstructure of nerve scaffolds, which frequently result in suboptimal nerve regeneration and functional recovery.

Electrical signal is an attractive guiding cue for promoting axonal regeneration in nerve injury repair. Despite the beneficial effect of ES on nerve regeneration which has been widely reported in animal models of crush injury [4], transected injury [5], and short femoral nerve defects [6,7], the application of ES in the treatment of large nerve defects has been rare. In our previous study, we introduced electrical cues at the local site of a conductive scaffold to establish an electrical environment between a large nerve defect [8]. The electrical environment between a lengthy nerve defect has been shown to be beneficial for nerve regeneration and functional recovery. It has to be realized that no cells or neurotrophic factors were introduced into the conductive scaffold. Therefore, it raises the possibility that nerve regeneration and functional recovery might be further enhanced by ES applied to the local site of a conductive scaffold with cell seeded. Before this attempt, one

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important issue which needs to be clarified is the effect of ES on seed cells through conductive scaffold.

OEC, as one of the classic seed cells [9–12], is a candidate cell to be combined with conductive graft because its migratory potential and ability to penetrate glial scars is higher in contrast to Schwann cells [13,14]. In addition, OECs can create a suitable microenvironment to enhance axonal regeneration and produce myelin after transplantation [15]. This makes OECs an attractive addition to artificial nerve grafts. Thus far, the effect of ES on OECs *via* conductive composite has not been investigated.

In previous studies, polypyrrole (PPy) has been widely studied because of its high conductivity and biocompatibility. It has been shown that PPy can be used for testing the blood lithium levels of patients being treated for bipolar disorder [16]. In addition, PPy has gained an increasing interest in the area of biomaterials for tissue regeneration over the last decade [17–20]. However, PPy has poor mechanical properties, such as being weak, brittle and nondegradable, which make it unsuitable for applications in nerve regeneration [38]. Composite materials that incorporate a small amount of PPy with another polymer that has suitable material properties can overcome these limitations. In our previous studies, an electrically conductive, biodegradable composite based on PPy and chitosan was developed [21,22]. In this conductive composite, a small amount of PPy (2.5% w/w) was incorporated into the chitosan matrix to obtain the desired conductivity while maintaining the flexibility and biocompatibility of chitosan, making the biodegradable conductive PPy/chitosan composite attractive in neural tissue engineering. Therefore, the present study was designed to investigate the interactions between OECs and conductive PPy/chitosan, and further examine the possible regulatory effect of ES on OECs through this conductive polymer.

2. Materials and methods

2.1. Preparation of PPy/chitosan membrane

PPy was synthesized using a microemulsion polymerization technique as described previously [21,22]. In brief, polypyrrole nanoparticles were synthesized from doubly distilled pyrrole monomers (98%; Sigma–Aldrich, US) in a water-in-oil emulsion system. Dodecyl benzenesulfonic acid sodium salt (SDBS; Sigma–Aldrich, US) was used as an emulsifier and $FeCl_3$ ($FeCl_3$: pyrrole, 2.3:1) as an oxidant. The resulting PPy particles were repeatedly washed with acetone and deionized water to thoroughly remove any residual emulsifier and $FeCl_2$. The resulting PPy powder was then suspended in a 0.5% acetic acid aqueous solution using an ultrasonic water bath before further use. To prepare PPy/chitosan composite (2.5% w/w PPy), chitosan powder (molecular weight >310 kD, degree of deacetylation ≈ 90%; Sigma–Aldrich, US) was first dissolved in a 1.0% (v/v) acetic acid aqueous solution to prepare a chitosan solution (1.5% w/v). Then the suspension of PPy was added into the chitosan solution with vigorous stirring at 65 °C for 4 h. The PPy/chitosan mixture with different PPy content (w/w, 1.0%, 2.5%, 4.0%, and 6.0%) was subsequently cast onto a plastic dish, and dried at room temperature in air for 24 h. The dry PPy/chitosan membranes were neutralized with 1.0% w/v NaOH aqueous solution for 30 min, washed with deionized water until neutrality was reached, and dried again at room temperature in air. The PPy/chitosan membranes were sterilized for cell culture use with ethylene dioxide gas at 37 °C according to standard industrial procedures. The conductivity of the PPy/chitosan mixture was measured with a standard four-point probe method at a constant current of 0.5 mA at ambient temperature. The membranes were dried to constant weight before the measurements were made. At least four specimens were measured for each sample and the recorded values were averaged. The PPy/chitosan membrane with PPy content of 2.5% was used in the degradation test. The PPy/chitosan membranes made of PPy/chitosan matrix (20 mg) were freeze-dried and sterilized. The sterilized PPy/chitosan membranes incubated in sterilized phosphate buffered saline (PBS) for different periods of time. At 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks and 6 weeks after incubation with PBS, the PPy/chitosan composite was freeze-dried again. The weight and conductivity of the dry PPy/chitosan composite were measured and recorded.

2.2. Isolation and purification of OECs

All procedures were conducted under a protocol reviewed and approved by the Institutional Ethical Committee of the Fourth Military Medical University. The Sprague–Dawley rats weighing 220 g around were deeply anesthetized with pentobarbitone and killed by decapitation. The head was immerged into the 0.5% iodine solution quickly for 3 min and then immerged into 75% alcohol for deiodination. The skull was opened, olfactory nerves were cut, olfactory bulbs were picked out and their caudal one-third of the bulb was removed and discarded along with as much white matter as possible to isolate the outer nerve layer. Tissue was finely minced with scalpel blades on culture dishes and incubated for 20 min in 0.25% trypsin/0.02% EDTA (Sigma–Aldrich, US) digestion solution at 37 °C on a rotary shaker in a CO_2 incubator. The digestion was ended by adding Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibico, US) medium with 20% fetal calf serum (FCS; Gibico, US), and the pellets were gently triturated with fire-polished pasture pipettes so as to successively reduce diameters and finally became free cells. The cell suspension was re-suspended and preplaced for 18 h in a culture flask at 37 °C in a 5% CO_2 incubator. Non adherent cells were gently washed off with DMEM/F12, and the cells were centrifuged and re-suspended in DMEM/F12 with 10% FCS. Then, the suspension was collected and inoculated into the poly-L-lysine (10 μg/mL; Sigma–Aldrich, US) coated culture plate and cultured in an incubator at 37 °C, 5% CO_2 for 3 days. Following that, half volume of medium was exchanged with medium containing Forskolin (100 mg/L; Cellagen Technology, US) and basic fibroblast growth factor (bFGF, 0.01 mg/L; Sigma–Aldrich, US). The total volume of medium was exchanged every 2–3 days during the next 14 days. The purity of the OECs cultures was determined by double immunofluorescent staining samples with NGFRp75 (ab62122; Abcam Inc., UK) and GFAP (ab49874; Abcam Inc., UK) antibody. These sample cultures were covered with glass slips with Citifluor containing DAPI (Invitrogen, US) and numbers of NGFRp75 and GFAP positive cells and DAPI-labeled cells were compared. The high purity (>95%) of the OECs was confirmed by images acquired from a fluorescence microscope (BX-51, Olympus, Japan).

2.3. Electrical cell culture system

The conductive PPy/chitosan membrane (thickness, 0.4 mm) was cut into rectangular sections (27.9 × 7.8 cm). The membrane sections were fixed to the bottom of the custom-built electrical cell culture plate. In this custom-built electrical cell culture plate, two thin platinum electrodes were fixed at the opposite two ends of the rectangular conductive polymer by using a water tight seal to avoid the direct contact of electrodes and culture medium. The electrodes were connected to a direct current (DC) power which is capable of supplying constant direct current. The surface of conductive polymer exposed to cell culture was 27 × 7 cm^2. The electrodes did not contact the culture medium directly to avoid possible weak ionic current generated.

2.4. Electrical stimulation of OECs

When the cultures of OECs reached 95% confluence, they were digested with a 0.05% trypsin solution, washed and re-suspended in DMEM/F12 with 10% FCS. The cells were then seeded on the PPy/chitosan membranes and cultured for 24 h to allow settling and adhesion. Then lateral constant potential gradients (50 mV/mm, 100 mV/mm, 300 mV/mm, 600 mV/mm, 1000 mV/mm) was then applied to the cells (M + ES group) for 60 min through the membrane using the custom-built electrical cell culture system described above. We chose direct current stimulation in this study because it has been shown to regulate cell functions in many previous studies [18–20]. After ES, the culture medium was replaced with fresh DMEM/F12 with 10% FCS. The cells were incubated for an additional 12, 24, or 36 h (total culture time 36, 48, and 60 h). Cells cultured on conductive membranes without ES (M–ES) and on culture plates (CP) without conductive membranes served as controls.

2.5. Tolerance of OECs to ES

The OECs (1 × 10^6 cell/cm^2) were cultured on the conductive PPy/chitosan membranes for 24 h, and then lateral voltage gradients ranging from 50 to 1000 mV/mm were applied through the conductive polymers for 60 min. After ES, OECs were incubated for another 24 h, then trypsinized, re-suspended in 200 μL binding buffer (10^6 cells per tube), and then incubated with 10 μL Annexin V-FITC and 5 μL PI (BD Pharmingen™, US) for 15 min at room temperature. Samples were then diluted to 500 μL with binding buffer and the number of viable cells was determined by flow cytometry (BD FACS caliber, BD Bioscience). Samples were analyzed within 60 min after preparation.

2.6. Evaluation of OECs adhesion

The OECs attached to the conductive PPy/chitosan membranes were fixed with paraformaldehyde (4% in phosphate buffered saline, PBS) at room temperature for 30 min, washed three times with distilled water, and dehydrated with serial ethanol solutions. The specimens were dried under vacuum at room temperature, sputter-coated with gold, and then subjected to scanning electron microscopy (SEM, Hitachi S-3400N, Japan) at an accelerating voltage of 5 kV to examine the morphology of the cells.

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