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In vitro biocompatibility of chitosan-based materials to primary culture of hippocampal neurons

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Abstract The natural biomaterial chitosan has been widely used as a promising nerve guidance conduit material for peripheral nerve repair. This study aimed to investigate in vitro biocompatibility of chitosan to primarily cultured hippocampal neurons, one type of central nervous system (CNS) cells. The substrate made up of chitosan fibers or membranes was found to support the survival and growth of the attached hippocampal neurons by using light and electron microscopy as well as immunocytochemistry for neurofilament 200, growth-associated protein-43, microtubule-associated protein 2, β-tubulin III and synaptophysin. MTT assay indicated that the cell viability of hippocampal neurons in chitosan fiber or membrane extract was not significantly different from that in hydroxyapatite extract or plain neuronal medium, but significantly higher than that in organotin extract after culture for different times. Western analysis revealed that no significant difference in the protein level of growth-associated protein-43 and β-tubulin III was detected between hippocampal neurons cultured in chitosan extract and in plain neuronal culture medium. The results collectively demonstrate that chitosan is biocompatible to primary culture of hippocampal neurons without cytotoxic effects on cell phenotype and functions, raising a potential possibility of using chitosan for CNS therapy.

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1 Introduction

The adult mammalian central nervous system (CNS) does not spontaneously recover from injuries and degeneration. This fact has long encouraged the development of CNS therapy strategies. Recently, neural stem cell implantation and controlled release of neutrophic factors are suggested to be two promising approaches to contribute to CNS regeneration. Both approaches require the use of biomaterials to prepare either nerve guide conduits/scaffolds or delivery vehicles for inclusion of either implanted cells or released factors, respectively [1–3]. Obviously, the biomaterials suitable for these two applications should provide appropriate chemical and spatial microenvironment for cell adhesion, proliferation, differentiation and axon extension in CNS, namely, should have good biocompatibility with CNS cells or tissues.

Chitosan is the fully or partially deacetylated form of chitin, the second most abundant polysaccharide in nature next to cellulose [4]. Chitosan possesses favorable biological properties to benefit cellular adhesion and inhibition of scar formation [5–12], thus finding a range of applications in biomedical, especially tissue engineering, fields [13–16].

In previous studies, we have investigated the interaction of Schwann cells with chitosan fibers and membranes in vitro [17], and also reported that chitosan/PGA nerve guidance conduits were implanted into dog sciatic nerve injuries across a 30-mm-long defect with a positive outcome of regeneration [18]. In this study, we aimed at investigating the possibility of chitosan used as a candidate biomaterial for CNS therapy besides peripheral nerve repair. The primarily cultured hippocampal neurons of developing rat brains, which usually serve as a well-characterized cell model [19], were cultured on the substrate

made up of chitosan fibers or membranes for examining the biocompatibility of chitosan with CNS cells. The cells were also treated with chitosan extract for assessing, as per International Standards ISO 10993-5, the cytotoxicity of chitosan to CNS cells.

2 Materials and methods

2.1 Preparation of chitosan films or membranes

Chitosan was obtained from Nantong Xincheng Biochemical Company (Jiangsu, China) with deacetylation degree of 92.3% and average-molecular weight (M_w) of 2.8×10^4.

Chitosan fibers were prepared by wet-spinning procedure as our previously described [20]. The dope was prepared by dissolving 40 g of chitosan in 1 l of 2% (w/w) acetic acid solution. A laboratory scale extrusion unit, composed of a reservoir, a metering pump (2.4 cm^3/rev), and a spinneret (500 holes, 80 μm diameter), was used. By applying nitrogen of pressure 7×10^5 Pa, the chitosan solution was passed through a constant volume metering pump, and to a stainless steel spinneret, which was immersed in a coagulation bath containing a solution of 7% (w/w) NaOH and 10% (w/w) Na_2SO_4. After exiting the coagulation bath, the fiber was advanced into a 1-m-long bath pool containing hot water. The take-up rollers, drawing system, drying rollers and the winding up procedure were as described elsewhere [21]. The chitosan fibers were washed and dried by radiant heat.

For preparing chitosan membranes, 1 g of chitosan was dissolved in 50 ml 1% (w/w) acetic acid solution and stirred. The solution was spread on glass dishes and dried at 50°C in a drying oven. After the resulting membranes had dried, they were immersed in 1% (w/w) NaOH solution for 24 h to neutralize the remaining acetic acid, followed by washes with distilled water until neutral pH was obtained. The thickness of the dried membrane was 50 μm [17].

2.2 Preparation of chitosan fiber or membrane extract

After sterilization, the chitosan matrices, either 1 g of chitosan fibers or 60 cm^2 of chitosan membranes, were placed in an extraction container with addition of 10 ml neuronal culture medium (Neurobasal medium with 1× B27 supplement, 0.5 mM glutamine, and 1× penicillin/streptomycin, Sigma, St. Louis, MI) and allowed to incubate at 37 ± 0.5°C for 72 ± 0.5 h. The prepared chitosan fiber or membrane extract had to be used within a 24-h period. The extracts of 0.1 g hydroxyapatite (from Sigma) and 0.1 g organotin (from Hongding Chemicals Company, Nantong, Jiangsu, China) in 10 ml of neuronal culture medium were prepared in the same manner, respectively.

2.3 Cell culture and treatment

Sprague-Dawley embryonic rats were obtained from the experimental animal center of Nantong University. All experimental procedures involving animals were conducted as per Institutional Animal Care guidelines and approved ethically by the administration committee of experimental animals, Jiangsu Province, China.

The rat hippocampal neurons were isolated by the method described previously with minor modifications [22, 23]. After E 18 embryonic rats were sacrificed by cervical dislocation under anesthesia, their brains were quickly removed and the hippocampi were harvested on a cold stage. The procured hippocampal tissues were mechanically and enzymatically dissociated into a single-cell suspension, which was then plated onto poly-lysine-coated plates at a density of 1×10^5 per cm^2 in DMEM supplemented with 10% F12 and 10% FBS (Gibco, Grand Island, NY) for 4 h. The medium was replaced by neuronal culture medium, and cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 7–8 days, the time required for maturation of hippocampal neurons. Half of the culture medium was replaced every 2 days. The glial content of cultured cells was measured to be only 0.5% of the total cell population.

Primarily cultured hippocampal neurons were seeded onto the poly-L-lysine (Sigma) coated coverslips, or the substrate made up of chitosan fibers (1.5 cm length) or chitosan membranes (8×8 mm^2 size) that had all been placed onto a 24-well culture plate, followed by soaking in plain neuronal culture medium. Another portion of primarily cultured hippocampal neurons was randomized into four groups for treatment with different mediums that were plain neuronal culture medium (positive control), hydroxyapatite extract (positive control), organotin extract (negative control) and chitosan extract, respectively.

2.4 Light and electron microscopy

The growth of hippocampal neurons cultured on the poly-lysine-coated coverslips, or the substrate made up of chitosan fibers or membranes was observed under an inverted microscope (Olympus, Tokyo, Japan) every day. Photographs were taken at regular intervals. In order to determine the average and total length of neurites for the hippocampal neurons cultured on the different substrates for 5 days, five fields were randomly selected for

measurements of each photograph with Q550 IW image analysis system (Leica Imaging Systems Ltd., Cambridge, England). All measurements were performed in triplicate.

After 3- or 7-day culture on the poly-lysine-coated coverslips, or the substrate made up of chitosan fibers or membranes, rat hippocampal neurons were washed twice with phosphate buffer saline (PBS, pH 7.2) and fixed in 4% glutaraldehyde. They were then post-fixed with 1% OsO_4, dehydrated stepwise in increasing concentrations of ethanol, and dried in a critical point drier (Hitachi, Tokyo, Japan), followed by coating with gold in a JFC-1100 unit (Jeol Inc., Japan) and observation under a scanning electron microscope (JEM-T300, Jeol Inc., Japan).

2.5 Immunocytochemistry

After 7-day incubation, the samples were washed three times with PBS and fixed with freshly prepared 4% paraformaldehyde solution in 0.01 M PBS for 30 min at room temperature. The fixed samples were incubated for 1 h in a solution containing 10% goat serum, 3% bovine serum albumin and 0.1% Triton-X 100 at room temperature to block nonspecific binding. Then, they were allowed to incubate with primary antibodies, i.e. goat anti-growth associated protein-43 (GAP-43, 1:200 dilution, Santa Cruz, CA), mouse monoclonal anti-microtubule-associated protein 2 (MAP2, 1:1,000 dilution, Sigma), rabbit anti-neurofilament (NF) 200 IgG fraction of antiserum (1:200 dilution, Sigma), rabbit anti-β-tubulin III (1:50 dilution, Sigma) and mouse monoclonal anti-synaptophysin (1:500 dilution, Sigma), overnight at 4°C in a humidified chamber. After being washed three times with PBS, the samples were further reacted with second antibodies: Cy3-labeled goat anti-rabbit IgG (1:200 dilution, Santa Cruz), FITC-labeled donkey anti-goat IgG (1:200 dilution, Santa Cruz) and TRITC-labeled donkey anti-mouse IgG (1:200 dilution, Santa Cruz) for 2 h at 37°C. The samples were washed three times with PBS, then incubated with 5 μg/ml Hoechst 33342 (Sigma) at room temperature for 15 min, mounted in fluorescent mounting medium and observed under a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany). Controls included leaving out the primary antibody and using non-labeled secondary antibodies to confirm inexistence of nonspecific binding.

2.6 MTT assay

A modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, in which the yellow MTT is reduced to a purple formazan by mitochondrial dehydrogenase in cells, was performed to assess the cell viability.

After 12-, 24-, 48- or 72-h incubation in different mediums, the viability of hippocampal neurons was assessed. Briefly, hippocampal neurons were washed three times with DMEM. The culture medium in each well of the plate was replaced with 100 μl DMEM and 25 μl MTT (5 mg/ml in PBS). After 4 h incubation at 37°C, the reaction was stopped by adding 100 μl lysis buffer (20% SDS in 50% *N*,*N*-dimethylformamide, pH 4.7). After 20 h incubation at 37°C, the supernatant of all wells were respectively aspirated out to be measured photometrically at 570 nm (OD_{570}) with an ElX-800 Microelisa reader (Bio-Tek Inc., Winooski, VT).

2.7 Western blot analysis

After 12-, 24-, 48- or 72-h culture either in neuronal culture medium or chitosan extract, the hippocampal neurons were washed with PBS and lysed with lysis buffer containing protease inhibitors (Promega, Madison, WI). Protein concentration was detected by BCA method (calibrated on bovine serum albumin) to maintain the same loads. Protein extracts were heat denatured at 100°C for 10 min, electrophoretically separated on a 10% SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBST buffer (50 mM Tris–HCl, 100 mM NaCl, and 0.1% Tween-20, pH 7.4) and incubated with a 1:500 dilution of goat anti-GAP43 polyclonal antibody and 1:1,500 dilution of rabbit anti β-tubulin III antibody in 5% non-fat dry milk in TBST buffer at 4°C overnight. The membranes were washed with TBST buffer (5 min × 3), and further incubated with a 1:5,000 dilution of donkey anti-goat IgG and 1:10,000 dilution of donkey anti-rabbit IgG at room temperature for 2 h. After the membrane was washed, the image was scanned with Odyssey (LI-COR, Lincoln, NE), and the data of optical density were analyzed using PDQuest 7.2.0 software (Bio-Rad).GAPDH (1:500) was used as an internal control.

2.8 Statistical analysis

At least three repetitive tests were performed, and all data were expressed with means ± SEM. A one-way ANOVA with the Stata 6.0 software package (Stata Corp., College Station, TX) was used to conduct statistical analysis, and statistical significance was accepted at the probability level $P < 0.05$.

3 Results

3.1 Hippocampal neurons onto chitosan fibers or membranes

Light microscopy was used for visualizing the cell growth of hippocampal neurons cultured on the poly-lysine-coated coverslips, or the substrate made up of chitosan fibers or membranes. After 3-day culture on the substrate made up of chitosan fibers, hippocampal neurons were oval or spindle in shape with neurites adhering to and extending along the fiber, and after 7-day culture the cells spiraled tightly along chitin fibers to form neural networks (Fig. 1c, d). On the other hand, hippocampal neurons were found to adhere to chitosan membranes immediately after inoculation, and the cells showed a spherical shape with smooth surface and obvious refraction. After 3-day culture, the neurites of hippocampal neurons adhered to and extended along the substrate (Fig. 1e), and after 7-day culture the cells with long and dense neurites were found to form neural networks (Fig. 1f). Moreover, the cell growth on the chitosan-based substrate was noted to be similar to that on the poly-lysine-coated coverslips (Fig. 1a, b).

After 5-day culture, the neurite outgrowth of hippocampal neurons cultured on the poly-lysine-coated coverslips (Fig. 2a) and the substrate made up of chitosan membranes (Fig. 2b) was found to be comparable to each other. This result was further evidenced by the comparisons in the average (Fig. 2c) and total (Fig. 2d) length of neuritis for hippocampal neurons cultured on the two different substrates without significant difference detected.

We also performed scanning electron microscopy to examine the cell growth of hippocampal neurons on the poly-lysine-coated coverslips, chitosan fibers or membranes. After 3-day culture, hippocampal neurons exhibited either a trigonal pyramidal shape or a spindle shape, and

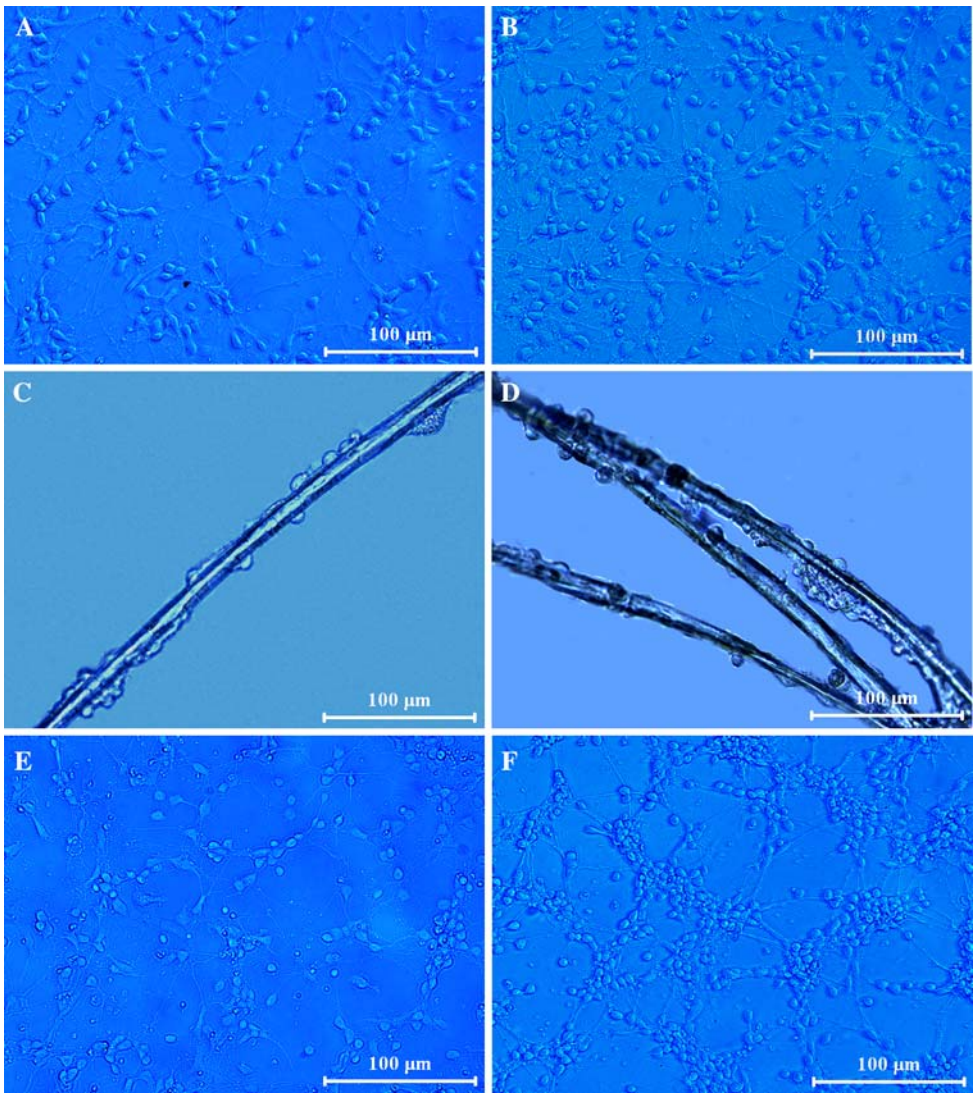

Fig. 1 Light micrographs of hippocampal neurons cultured on the poly-lysine-coated coverslips (**a**, **b**), the substrate made up of chitosan fibers (**c**, **d**) or membranes (**e**, **f**) for 3 days (**a**, **c**, **e**) or 7 days (**b**, **d**, **f**), respectively. Scale bar: 100 μm

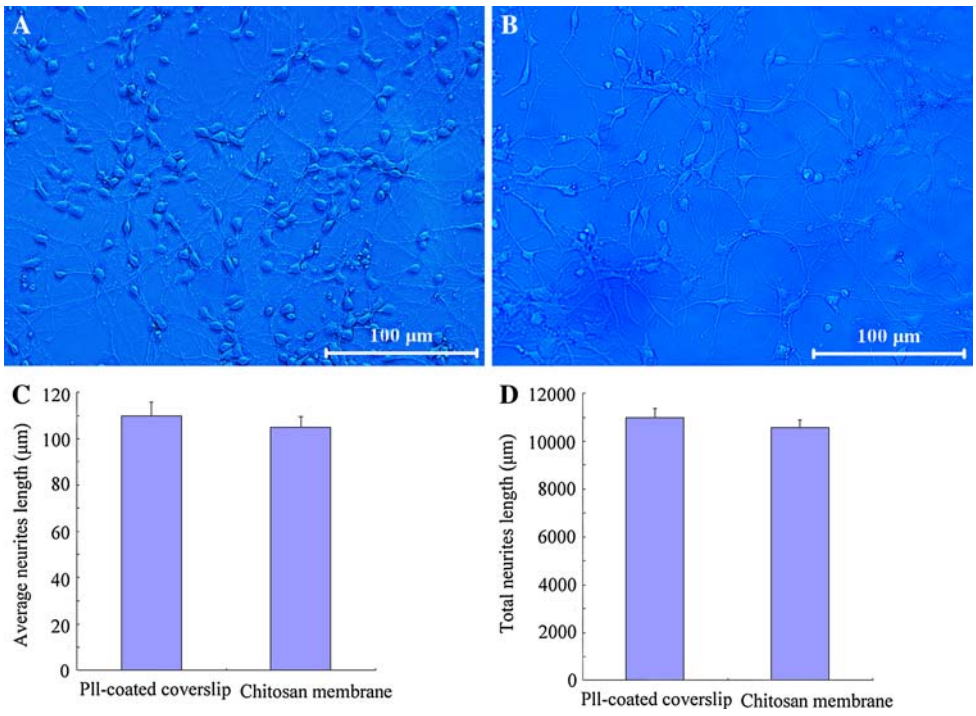

Fig. 2 Light micrographs of hippocampal neurons after 5-day culture on the poly-lysine-coated coverslips (**a**) and the substrate made up of chitosan membranes (**b**), respectively. The histograms showing the average (**c**) or total (**d**) length of neurites for hippocampal neurons cultured on poly-lysine (*Pll*) coated coverslips and the substrate made up of chitosan membranes for 5 days. No significant differences in either length was found between two different substrates ($P > 0.05$)

tightly attached to chitosan fibers with long neurites extending along the fibers (Fig. 3c); after 7-day culture, hippocampal neurons took compact arrangements of either side-by-side or end-to-end configuration, forming a three-dimensional cell chains (Fig. 3d). On the other hand, after 3-day culture, hippocampal neurons adherent to chitosan membranes were oval- or spindle-shaped, and they connected to each other via extending neuritis (Fig. 3e); and after 7-day culture the cells with long and dense axons and dendrites took compact arrangements, and they interweaved mutually to form neural networks (Fig. 3f). Also interestingly, the above-mentioned morphological features were close to those for hippocampal neurons cultured under normal conditions (Fig. 3a, b).

Taken together, histological observations revealed that primarily cultured hippocampal neurons were able to adhere to and grow on the chitosan substrate, and there were few visible differences in cell behaviors between hippocampal neurons on the poly-lysine coated coverslips and on the chitosan-based substrate, despite geometric divergence of the different substrates.

3.2 Immunocytochemical observation of hippocampal neurons onto chitosan fibers or membranes

After 7-day culture on the chitosan-based substrates, the hippocampal neurons underwent immunocytochemistry with antibodies against GAP-43, MAP2, NF200, β-tubulin III and synaptophysin, respectively, because the expression of these five important neuronal marker proteins presents an effective standard for biocompatibility evaluation of chitosan-based substrates.

GAP-43, an axonal membrane protein, is involved in the neuronal outgrowth and synaptic plasticity of developing and regenerating neurons [24–26], and MAP2, as the major microtubule associated protein of brain tissue, promotes microtubule assembly and forms side-arms on microtubules [27]. As was shown by GAP-43 (green) and MAP2 (red) double-labeled staining (see color figure online), in contrast to the situation of hippocampal neurons cultured on the poly-lysine coated coverslips (Fig. 4A), the cells (double-stained) were noted to tightly spiral around chitosan fibers, while axons (stained by GAP-43) and dendrites (stained by MAP2) were found to enwrap chitosan fibers to form neural networks (Fig. 4B). Anti-NF immunocytochemistry following 7-day culture demonstrated that NF immunopositive hippocampal neurons attached to and encircled the chitosan fibers as characterized by the relatively strong red fluorescence emission (Fig. 4C), on the other hand, NF immunopositive hippocampal neurons expanded on the chitosan membranes, interconnecting to form cell nets (Fig. 4D).

Beta-tubulin III, as a member of tubulin family, is found in the brain and root ganglia and localized to neurons in the central and peripheral nervous system, where its expression seems to increase during axonal outgrowth [28]. After 7-day culture on chitosan fibers, β-tubulin III immunopositive hippocampal neurons were observed to attach to and encircled the chitosan fibers as characterized by the

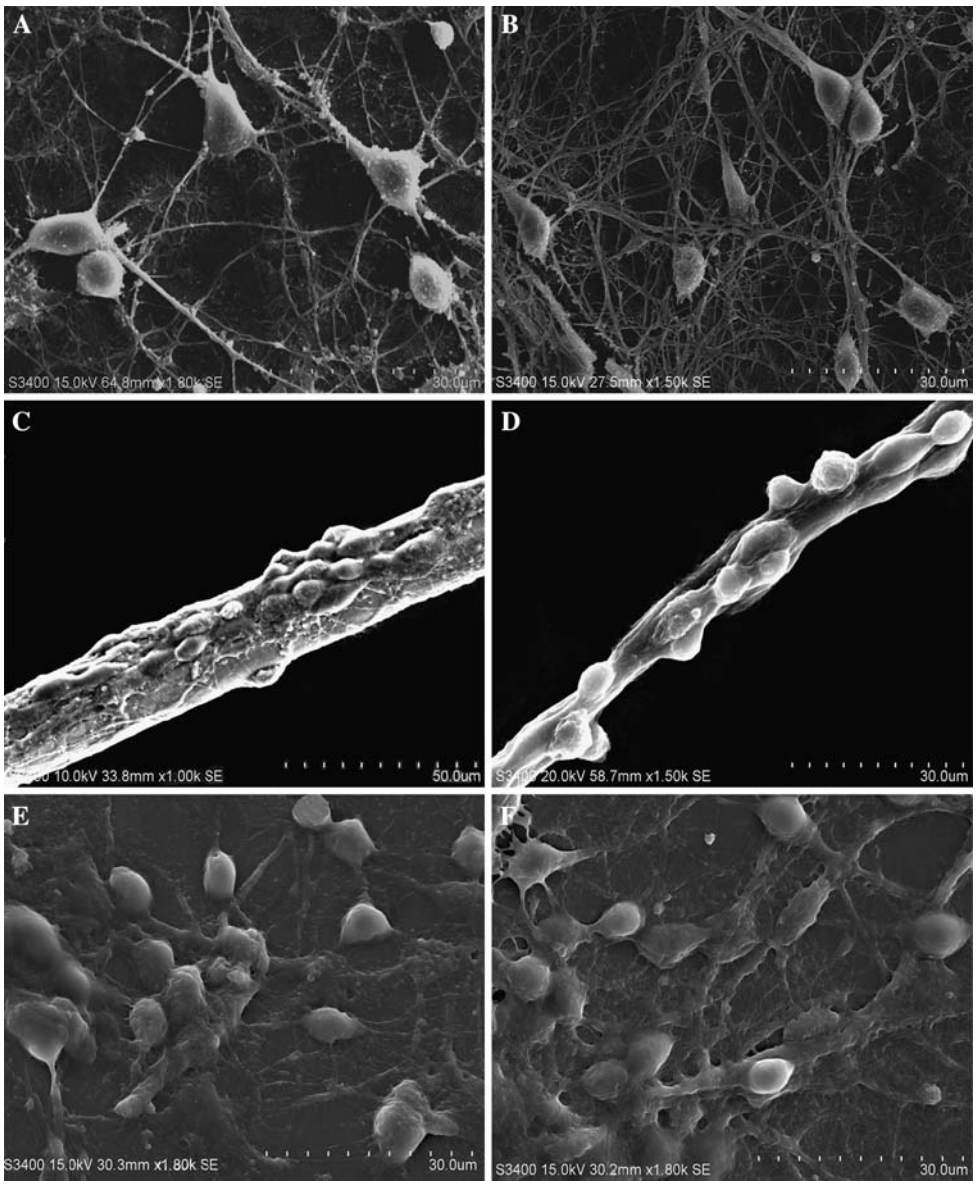

Fig. 3 Scanning electron micrographs of hippocampal neurons cultured on the poly-lysine-coated coverslips (**a**, **b**), the substrate made up of chitosan fibers (**c**, **d**) or membranes (**e**, **f**) for 3 days (**a**, **c**, **e**) or 7 days (**b**, **d**, **f**), respectively. Scale bar: 50 (**c**), 30 (**a**, **b**, **d**, **e**, **f**) μm

relatively strong red fluorescence emission (Fig. 5A), while β-tubulin III positive neurons were found to expand on the chitosan membranes, thus interconnecting to form cell nets (Fig. 5B). Synaptophysin is the most abundant integral membrane protein of synaptic vesicles and can be used as a mark protein of synaptic vesicles [29–32]. Anti-synaptophysin immunocytochemistry revealed that the expression and distribution of synaptophysin in hippocampal neurons cultured on the chitosan membranes (Fig. 5D) were not significantly different from those cultured on the poly-lysine coated coverslips (Fig. 5C).

Collectively, immunocytochemistry showed that neuron-associated protein molecules were expressed by hippocampal neurons cultured on the different substrates under investigation, thus specifying neuron identity of the cells. These data provide further evidence that the chitosan fibers or membranes, as well as the poly-lysine coated coverslips, are biocompatible to hippocampal neurons in vitro.

3.3 Cell viability test for hippocampal neurons cultured in chitosan extract

To determine whether chitosan fiber or membrane extract were cytotoxic to CNS cells, we performed MTT tests, and found that the cell viability of hippocampal neurons cultured in chitosan fiber or membrane extract was not significantly different from that in plain neuronal medium or in hydroxyapatite extract after 12-, 24-, 48-, and 72-h culture, while the viability of hippocampal neurons

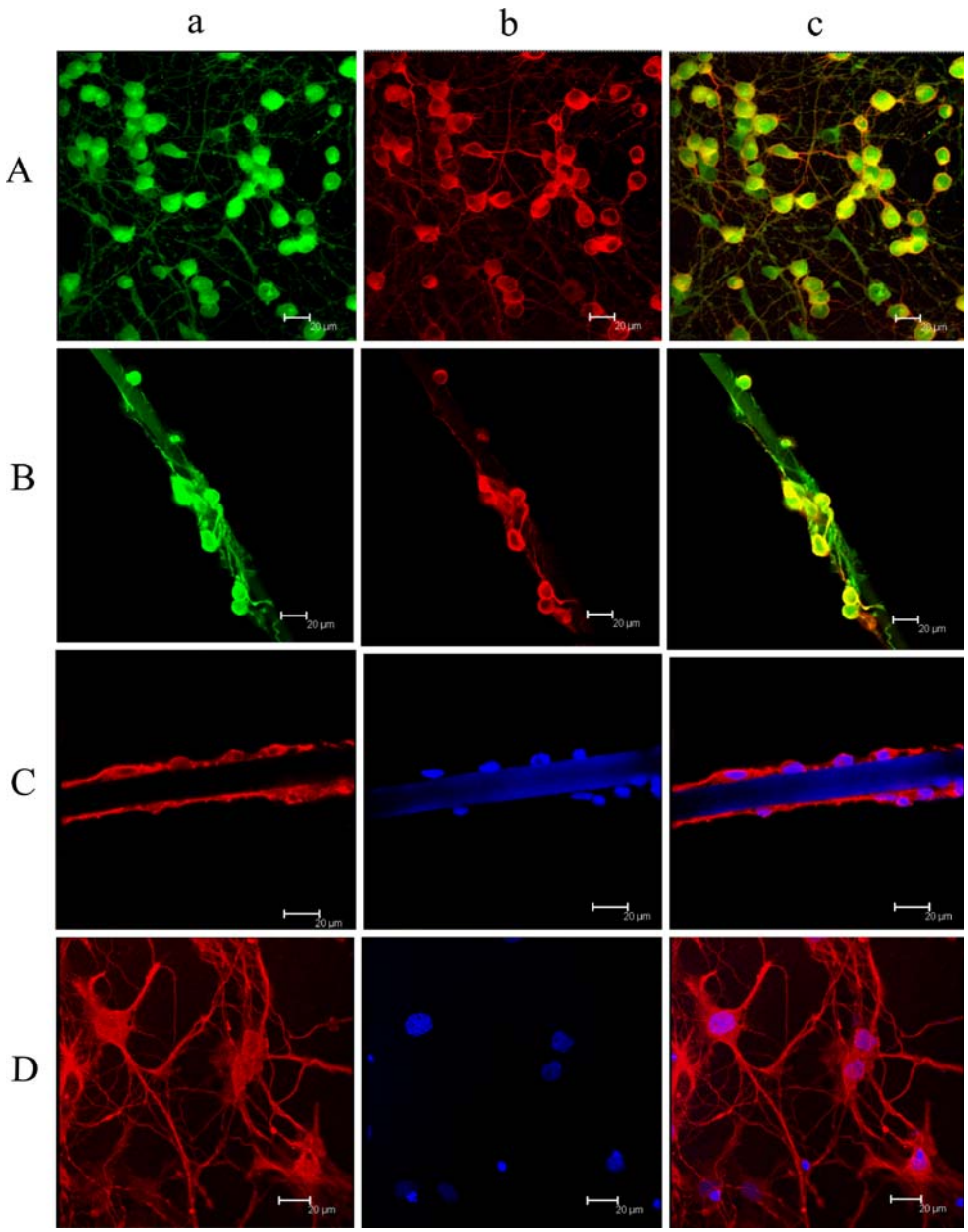

Fig. 4 GAP-43 and MAP2 double-labeled staining of hippocampal neurons after 7-day culture on the poly-lysine-coated coverslips (**A**) or the chitosan fibers (**B**), in which GAP-43 immunopositive cell bodies and axons were seen in (**a**), MAP2 immunopositive cell bodies and dendrites were seen in (**b**), and (**c**) is the merge of (**a**) and (**b**). Anti-NF-200 immunocytochemistry and Hoechst 33342 staining of hippocampal neurons after 7-day culture on the substrate made up of chitosan fibers (**C**) or membranes (**D**), in which NF immunopositive neurons were seen in (**a**), Hoechst-labeled cell nuclei of neurons were seen in (**b**), and (**c**) is the merge of (**a**) and (**b**). Scale bar: 20 μm

cultured in organotin extract, a negative control used in an international accepted standard for cytotoxicity test, was significantly lower than that in each of other culture mediums at all time points of culture (Fig. 6).

3.4 Expression of GAP-43 and β-tubulin III by hippocampal neurons treated with chitosan extract

Western blot analysis was conducted to determine the expression levels of GAP-43 and β-tubulin III proteins in hippocampal neurons that were cultured in plain neuronal medium and chitosan fiber or membrane extract for 12-, 24-, 48- and 72-h, respectively. No significant differences were detected between the two mediums (Fig. 7), suggesting no inhibitory effects of chitosan substrate on the biological functions of primary culture of hippocampal neurons.

4 Conclusion

Chitosan has proved to have good in vitro biocompatibility with peripheral nerve cells or tissues, and can serve as a promising nerve conduit material to promote peripheral nerve regeneration. In this study, primary culture of hippocampal neurons was used as a cell model to evaluate in

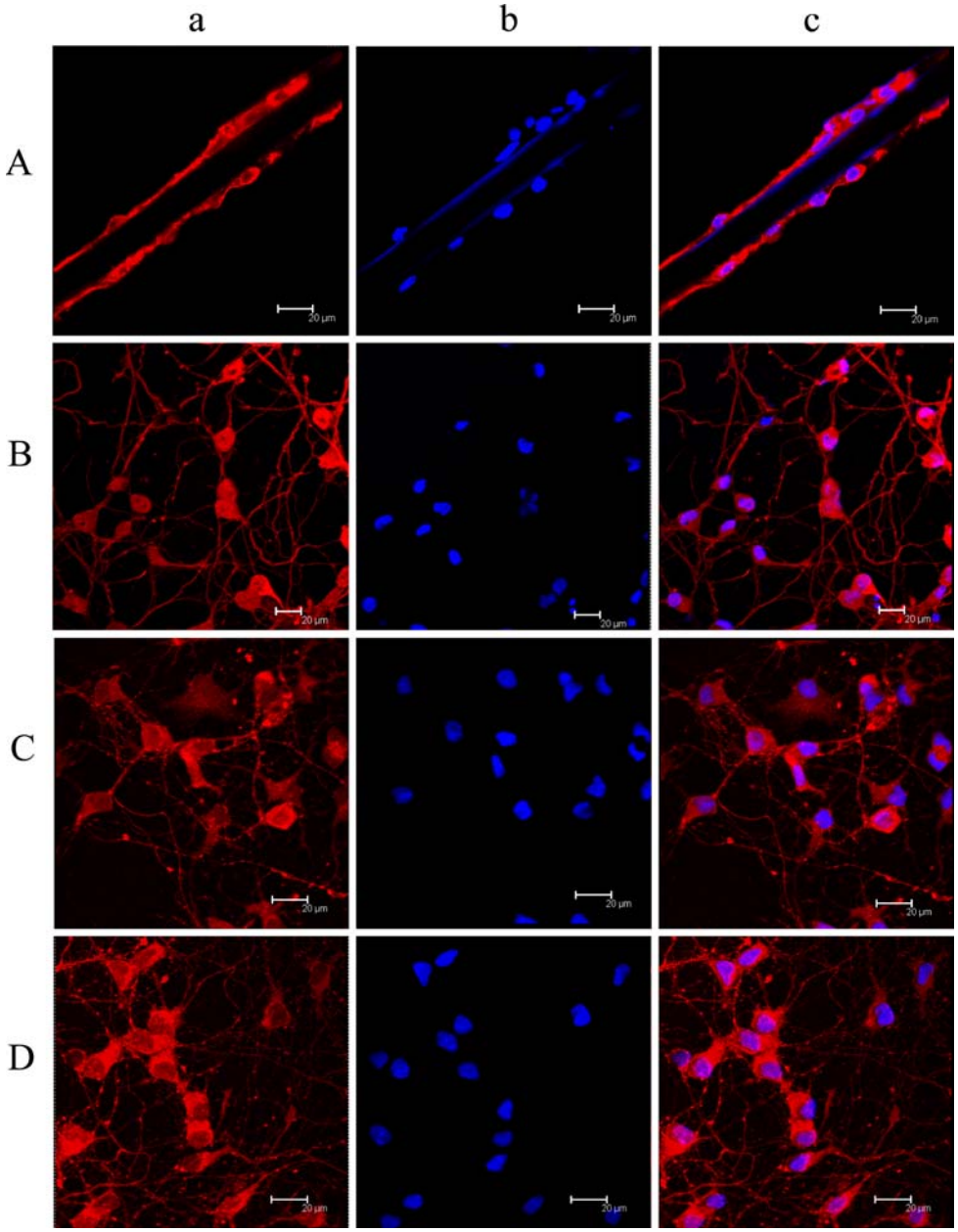

Fig. 5 Anti-β-tubulin III and Hoechst 33342 staining of hippocampal neurons after 7-day culture on the substrate made up of chitosan fibers (**A**) or membranes (**B**), in which β-tubulin III immunopositive neurons were seen in (**a**), Hoechst-labeled cell nuclei of neurons were seen in (**b**), and (**c**) is the merge of (**a**) and (**b**). Anti-synaptophysin and Hoechst 33342 staining of hippocampal neurons after 7-day culture on the poly-lysine-coated coverslips (**C**) or the substrate made up of chitosan membranes (**D**), in which synaptophysin immunopositive neurons were seen in (**a**), Hoechst-labeled cell nuclei of neurons were seen in (**b**), and (**c**) is the merge of (**a**) and (**b**)

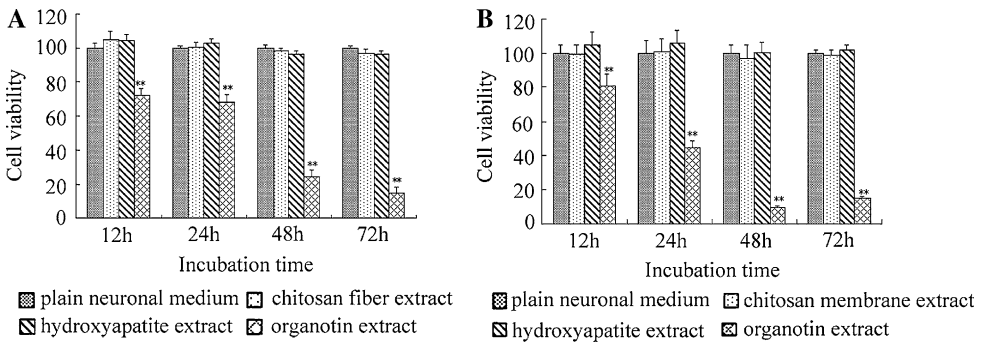

Fig. 6 The changes in the cell viability of hippocampal neurons, measured by MTT assay, after they were cultured in chitosan fiber (**a**) or membrane (**b**) extract, hydroxyapatite extract (**a**, **b**), plain neuronal medium (**a**, **b**) and organotin extract (**a**, **b**) for 12, 24, 48 or 72 h, respectively. The cell viability was expressed as the value relative to that in plain neuronal culture medium. $^{**}P < 0.01$ vs. other three mediums

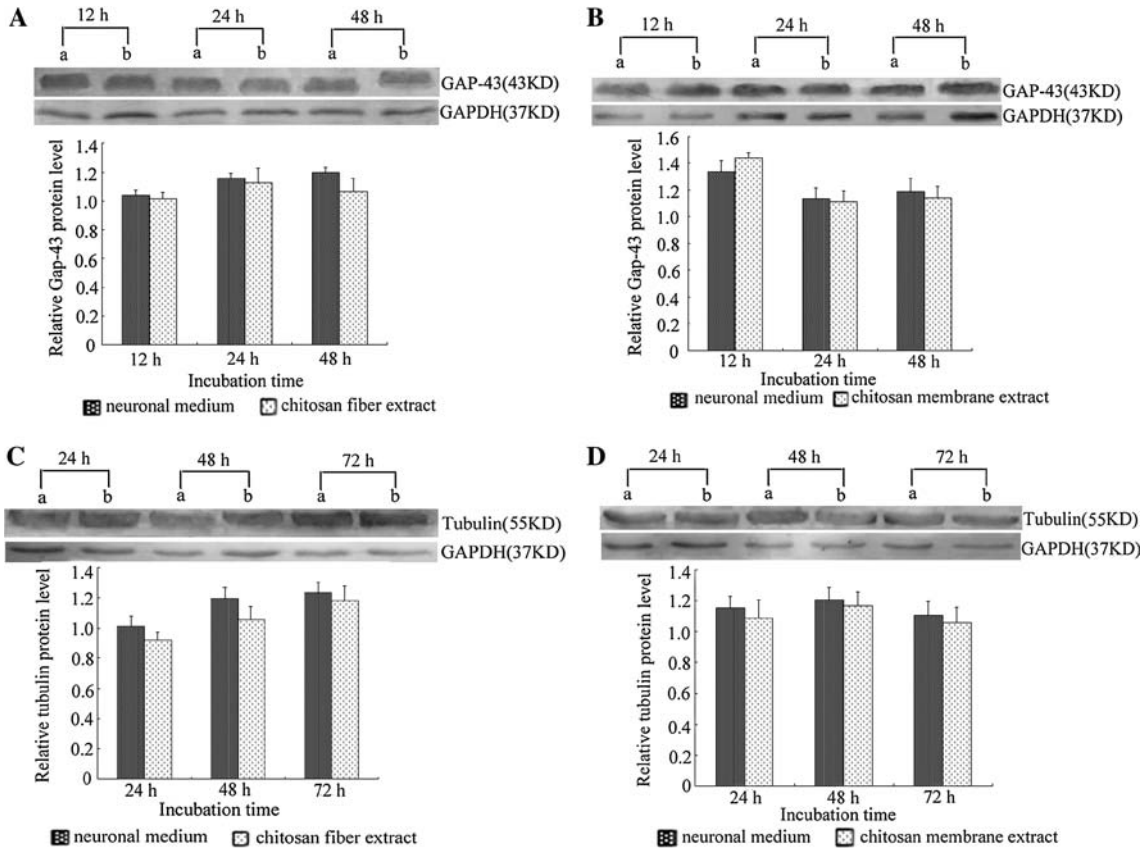

Fig. 7 The protein levels (relative to GAPDH) of GAP-43 (**a**, **b**) or Tubulin (**c**, **d**) in the hippocampal neurons that were treated with plain neuronal culture medium (**a**), chitosan fiber extract (**a** and **c**, **b**) or chitosan membrane (**b** and **d**, **b**) extract for 12, 24, 48, and 72 h, respectively. There were no significant differences between the two mediums at the same time point ($P > 0.05$). Also shown are the representative Western blots

vitro biocompatibility of chitosan materials to CNS cells. A series of in vitro tests confirm that chitosan is biocompatible to primary culture of hippocampal neurons without cytotoxic effects on cell phenotype and functions, raising a possibility of expanding chitosan application areas from peripheral nerve repair to CNS therapy.

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