In vitro evaluation of the compatibility of a novel collagen-heparan sulfate biological scaffold with olfactory ensheathing cells

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Background Stroke and traumatic injury to the nerve system may trigger axonal destruction and the formation of scar tissue, cystic cavitations and physical gaps. Olfactory ensheathing cells (OECs) can secrete neurotrophic factors to promote neurite growth and thus act as a prime candidate for autologous transplantation. Biological scaffolds can provide a robust delivery vehicle to injured nerve tissue for neural cell transplantation strategies, owing to the porous three-dimensional structures (3D). So transplantation of the purposeful cells seeded scaffolds may be a promising method for nerve tissue repair. This study aimed to evaluate the compatibility of a novel collagen-heparan sulfate biological scaffold with olfactory ensheathing cells in vitro.

Methods Collagen-heparan sulfate (CHS) biological scaffolds were made, and then the scaffolds and OECs were co-cultured in vitro. The viability of OECs was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay at days 1, 3, 5 and 7. Statistical analysis was evaluated by student's t test. Significance was accepted at P <0.05. OECs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and the CFSE-labeled OECs were seeded into CHS scaffolds. The attachment and growth of OECs in CHS scaffolds were observed and traced directly by fluorescent microscopy and environmental scanning electron microscope (ESEM).

Results CHS biological scaffolds had steady porous 3D structures and no cytotoxicity to OECs (F=0.14, P=0.9330). CHS biological scaffolds were good bridging materials for OECs attachment and proliferation, and they promoted the axonal growth.

Conclusion The compatibility of CHS biological scaffolds with OECs is pretty good and CHS biological scaffold is a promising cell carrier for the implantation of OECs in nerve tissue bioengineering.

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assessed CHS biological scaffold in terms of its ability to support cell attachment, viability, proliferation and of its ability to support axonal growth of OECs.

METHODS

Preparation of CHS biological scaffolds suspension

The CHS biological scaffolds were composed of type I collagen isolated from bovine achilles tendon (Sigma, USA), type IV collagen isolated from bovine placenta (Abcam, UK) and HS isolated from porcine intestinal mucosa (Sigma). The three components purchased as sterile lyophilized powder were dissolved to final concentrations of 10, 1.5 and 0.1 mg/ml respectively in a solution of 0.05 mol/L sterile acetic acid (pH 3–4). This was an important difference from the study of Wang et al.\textsuperscript{18} that their final concentrations of three components were all twice as much as ours. Then three components were mixed at 4ºC, 5000 r/min in a blender (78HW-I, Jiangsu Jintan Co., China). The CHS suspension was then degassed under vacuum (50 mTorr) at room temperature for 60 minutes to remove air bubbles introduced by mixing and was stored at 4ºC overnight until use and it should be degassed again right before use.\textsuperscript{18,19}

Fabrication of porous CHS biological scaffolds

After being degassed, the CHS suspension was enclosed into a silica gel pipe (10-cm length, 3-mm diameter) and stored at −80ºC for 2 hours. Then the pipe was cut into cylindrical columns (2-cm length, 3-mm diameter) and immediately removed to a stainless-steel tray (10 cm × 10 cm) which was then placed into the chamber of a freeze-dryer (FD-1-50, Beijing Boyikang Co., China) at −30ºC. The temperature of the freeze-dryer shelf and chamber was maintained at a constant rate to the final temperature of freezing (−30ºC, 100 mTorr) for 24 hours to produce the porous CHS scaffolds.\textsuperscript{19} Cross-linking and sterilization of CHS scaffolds were made with ultraviolet rays (500 µW/m²) for 2 hours. In the end, the sterile desiccated CHS scaffolds were stored at 4ºC.

Primary culture and purification of OECs

The animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Tongji Medical College. All efforts were made to minimize the number of animals used and their suffering in our study. Ten 3-day old Wistar rats were deeply anesthetized with CO₂ and sacrificed to obtain olfactory bulbs. After removal of the meninges, the external olfactory nerve layers were microdissected and dissociated at 37 ºC for 15 minutes twice in a solution of 0.25% trypsin and 0.03% collagenase. Trypsinization was stopped by adding DMEM/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and the digested tissue was mechanically dissociated by trituration. The cell suspension was put through a sterile gauze (74-µm pore size) and then centrifuged at 1000 r/min for 10 minutes. Cells were resuspended and plated in flasks fed with fresh complete medium, DMEM/F12 supplemented with 10% fetal bovine serum, forskolin (20 µmol/l) (Sigma), bovine pituitary extract (20 µg/ml) (Sigma), penicillin (100 U/ml) and streptomycin (100 U/ml). After 24 hours, the culture was treated with 10⁻⁵ mol/L cytosine arabinoside for 48 hours. In the last passage OECs were plated on the flasks coated with 10 µg/ml poly-L-lysine and cultured in complete medium supplemented at a final density of $5 \times 10^6$ cells/ml. By 10 days, the primary cultures were then passaged again once a week for 3–4 weeks before being harvested for use.

Fluorescence immunocytochemical analysis

Fluorescence immunocytochemical analyses of the OECs from passage 2 were performed using two antibodies: nerve growth factor receptor (NGFR p75) and glial fibrillary acid protein (GFAP). Cells were fixed with 4% paraformaldehyde, pH 7.5, for 15 minutes at 37 ºC, and permeabilized by 0.05% Triton X-100 in PBS and 1% fetal bovine serum for 60 minutes. Then they were incubated with polyclonal rabbit anti-p75 NGFR antibody (1:200) (Sigma) and mouse monoclonal anti-GFAP (1:200) (Santa Cruz, USA) and then incubated with FITC-labeled goat anti-rabbit IgG antibody (1:100) (Sigma) and Cy3-labeled rabbit anti-mouse antibody (1:100) (Sigma). All antibodies were diluted in PBS with 0.5% fetal bovine serum. In all assays, controls were performed by incubating cells with only the secondary antibodies. The treated sections were scanned with a fluorescence microscope (Olympus, Japan).

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OECs

OECs labeled by CFSE (Sigma) can be observed and traced directly under the fluorescent microscopy everyday. Suspensions of the OECs from passage 2 were centrifuged at 1000 r/min for 10 minutes and resuspended in PBS for staining. Then, 5 mmol/L CFSE was diluted to 2 mmol/L in PBS.\textsuperscript{20} CFSE-labeled cells were analyzed by flow cytometry.

Cytotoxicity assessment of the scaffolds using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) (MTT) assay

The cytotoxicity of scaffolds for OECs was detected by MTT assay. The cells (2×10⁴/200 µl) were plated in 96-well plates and there were 4 groups: cells control group (group A), cells plus scaffolds group (one scaffold per well) (group B), scaffolds group (one scaffold per well) (group C) and blank group (group D). Cells were cultured for 1, 3, 5 and 7 days, and washed once with PBS before adding 0.1 ml MTT solution (0.5 mg/ml, Sigma) to each well. After incubation for 4 hours at 37ºC, the supernatant was removed and 150 µl dimethylsulfoxide (Sigma) was added into each well, stirring for 15 minutes on a microtiter plate shaker. The absorbance (A₅₇₀) of the cell lysates was measured at 570 nm by a microplate reader (the reference wave length was at 630 nm).\textsuperscript{21}
Cell adhesion under environmental scanning electron microscope (ESEM)

ESEM was used to analyze the fine structure of non-seeded and seeded scaffolds. Seeded samples were placed into high density (20,000 cells/µl) cell suspensions of CFSE-labeled OECs for 2 hours and non-seeded samples were put into fresh complete medium without cells. The scaffolds and OECs were co-cultured for 7 days. Then, seeded scaffolds were fixed with 4% glutaraldehyde for 4 hours, cut along longitudinal axis using a razor blade, and dehydrated in acetone using a critical point dryer (completed by Medical College of Wuhan University). Non-seeded samples were untreated. The prepared specimens were mounted on stubs and sputter coated with gold and then loaded into an ESEM Quanta 200 scanning electron microscope (FEI, Netherlands), viewed under an accelerating voltage of 5 kV.8

Statistical analysis

All data were expressed as the mean ± standard error (SE) and processed with SPSS 13.0 software (SPSS, Chicago, USA). Statistical analysis was evaluated by student’s t test. Significance was accepted at $P < 0.05$.

RESULTS

Porous 3D structure analysis of CHS biological scaffolds

The CHS biological scaffolds were produced in the shape of cylindrical columns (2–3 cm length, 3-mm diameter, in white color) (Figure 1A). ESEM examination of the non-seeded scaffolds showed many longitudinal oriented microtubules and a high degree of porosity (Figure 1B–1F). Adjacent channels were linked to each other through a large number of windows on the channel walls (Figure 1B–1E) which were smooth and continuous (Figure 1D). Transverse sections of the scaffold showed the wasp-nest-like appearance (Figure 1E). The channel in the centre was straight and tightly packed (Figure 1F).

ESEM images of transverse sections were used to measure the diameter of channels of the CHS scaffold, inferring the typical channel size distribution (Figure 2). It revealed a broad distribution of the channel size ranging from 35 to 107 µm in diameter ((64.63±3.13) µm). But most of the channel size concentrated upon 50 to 70 µm in diameter.

Culture of neonatal rat OECs and immunocytochemical analysis

OECs were identified in culture by their characteristic morphology (bi- or multipolar cells with long processes, small nucleus, and reduced cytoplasm) and the expression of NGFR p75 and GFAP. Under the phase-contrast microscopy, the OECs of passage 2 were fusiform and pellucid at day 7 (Figure 3). Double fluorescence immunocytochemical analysis of OECs showed that at least 95% of cells co-expressed NGFRp75 and GFAP (Figure 4), suggesting the OECs cultured could be used for further study.
Cytotoxicity assessment of the scaffolds by using MTT assay
A570 values detected by MTT were different at days 1, 3, 5 and 7 but at the same point, there was no significant difference between groups A and B (P >0.05, Figure 5, Table). It suggested that CHS biological scaffolds had no cytotoxicity for OECs. In our study, OECs always kept fine viability when co-cultured with CHS biological scaffolds. The experiment was repeated at least twice.

![Figure 5](#)

**Table.** A570 values detected by MTT of groups A and B

<table>
<thead>
<tr>
<th>Days</th>
<th>A570 Group A</th>
<th>A570 Group B</th>
<th>t values</th>
<th>P values</th>
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<tr>
<td>1</td>
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<td>0.2540±0.0057</td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td>5</td>
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<td>0.4065±0.0225</td>
<td>0.03</td>
<td>0.9763</td>
</tr>
<tr>
<td>7</td>
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<td>0.2425±0.0185</td>
<td>0.38</td>
<td>0.7384</td>
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</tbody>
</table>

Cell adhesion and growth in the scaffolds
CFSE-labeled OECs were certified by ways of flow cytometry. Flow cytometry analysis showed that 99.8% of cells were labeled by CFSE. OECs were stained in green with CFSE when they were observed under the fluorescent microscopy. CFSE-labeled OECs were observed under the fluorescent microscopy. CFSE-labeled OECs, seeded into the scaffolds, were tracked directly under the fluorescent microscopy (Figure 6). ESEM images showed that OECs had adhered to the scaffold and fine long axons had grown out after co-culturing for 7 days (Figure 7). Some axons crawled along and attached to the walls, and others suspended within the lumen and branched at the walls of the scaffold, as vines. It indicated that OECs could survive, adhere, proliferate, grow out their axons and keep high cell viability in the CHS scaffold.

DISCUSSION
OECs might be considered in therapeutic management of central nervous system injury and demyelinating diseases, for their functional restoration and neural plasticity and for their neuroprotective effect in neurodegenerative disorders. Transplantation of OECs has become an ongoing clinical therapeutic approach in recent years. But the efficacies of some transplantation of OECs are uncertain and even undesirable.22-25 At the site of lesion, both necrotic and apoptotic events are taking place, and the activated cascades and molecules released may compromise the survival of the implanted cells. Because of the poor micro-environment for cell survival, implanting cells alone will not result in optimum recovery of function and thus combined strategies should be paid more attention to.26 The transplantation of scaffold combined with desirable cells is a good example. Compatible biomaterials containing OECs may protect cells from dead signals in the case of extensive necrosis and may also create a bridge between stumps when there is tissue loss. In the present study we aim at developing this new OECs-based therapeutic tool as a combined strategy. It may become a hopeful intervention for the future.

In the present study we focused on the special steady 3D structure of CHS biological scaffolds, which had relative uniform aperture, high degree of porosity and many longitudinal oriented microtubules. Our scaffolds took on the optimum 3D structure because of incomplete dissolution of components, compared with the study of Wang et al.18 These contributed to the immobilization, attachment, proliferation and migration of transplanted cells, offering enough solid spaces and playing a guiding role. And we also demonstrated that CHS biological scaffold had excellent compatibility with OECs, which
was a neoteric scaffold. It could promote the attachment, viability and proliferation of OECs and support the axonal growth.

Besides, the unique components of CHS also play an important role in the growth and adhesion of OECs. Recently, collagen scaffold is very popular and has lots of advantages in tissue engineering.6,7 But few investigators consider the combination of materials. Most of the growth and adhesive proteins have heparin-binding domains which interact with HS. In all cases, their biochemical characteristics and biological activity will be altered after the domains bind to HS. It provides a foundation for developing functional biomaterials designed to promote cell adhesion and growth, with the specific nature of the cell surface receptor-ECM interactions.9,27 Furthermore, it has been shown that the attachment and growth of cells were promoted in the scaffolds, which was supported by our study and many other experiments.9,13,18,28 Therefore, biomaterials composed of HS can promote the adhesion and growth of cells. Pieper et al14 reported that the loading of CHS matrices with bFGF enhanced angiogenesis and generation of new tissue because of inducing an intense and prolonged tissue response. This may be not only due to the direct angiogenic and mitotic properties of bFGF, but also provide chemotactic stimuli for various cells including endothelial cells, fibroblasts and macrophages. When activated cells in the matrices, especially macrophages, release a plethora of growth factors with chemotactic, angiogenic and mitogenic activity, it may induce subsequent cellular influxes and formation of capillaries. Furthermore, after the CHS matrices with bFGF are implanted, a transitional inflammatory response and a foreign body reaction also contribute to the infiltration of cells and the release of various cytokines and growth factors. We used OECs in our experiment though bFGF did not be used. Pellitteri et al29 reported that OECs added some growth factors, such as bFGF, in the neuronal cultures or feeding hippocampal neurons with conditioned medium. This demonstrated that compatible CHS biological scaffold containing OECs could be an excellent combined strategy applying to the repair of tissue injury. In addition, the producing procedure of the CHS biological scaffold is not difficult and its shape is easy to control. It is reasonable to infer that it may become a promising novel and useful biological scaffold.

It is difficult to observe the state of implanted cells without any tracers in the solid scaffold. In our study, we used CFSE to label OECs. By doing this, we tracked the cells and adjusted the density of implanted cells by fluorescent microscope easily. On the whole, we produced a novel biological scaffold which has a good compatibility with OECs in vitro. Furthermore, as a combined strategy, the transplantation of CHS combined with OECs may be a good choice for the reconstruction of nerve function. But the time of cytotoxic assessment with MTT assay seemed not long enough in our present study. Yoshii et al30 reported that the death rate of the animals was increased at 12 weeks after implantation of collagen filaments. So characteristics of OECs-based CHS biological scaffolds need more in-depth study in vivo in our future research.

REFERENCES


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