Collagen/heparan sulfate porous scaffolds loaded with neural stem cells improve neurological function in a rat model of traumatic brain injury

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Abstract

One reason for the poor therapeutic effects of stem cell transplantation in traumatic brain injury is that exogenous neural stem cells cannot effectively migrate to the local injury site, resulting in poor adhesion and proliferation of neural stem cells at the injured area. To enhance the targeted delivery of exogenous stem cells to the injury site, cell therapy combined with neural tissue engineering technology is expected to become a new strategy for treating traumatic brain injury. Collagen/heparan sulfate porous scaffolds, prepared using a freeze-drying method, have stable physical and chemical properties. These scaffolds also have good cell biocompatibility because of their high porosity, which is suitable for the proliferation and migration of neural stem cells. In the present study, collagen/heparan sulfate porous scaffolds loaded with neural stem cells were used to treat a rat model of traumatic brain injury, which was established using the controlled cortical impact method. At 2 months after the implantation of collagen/heparan sulfate porous scaffolds loaded with neural stem cells, there was significantly improved regeneration of neurons, nerve fibers, synapses, and myelin sheaths in the injured brain tissue. Furthermore, brain edema and cell apoptosis were significantly reduced, and rat motor and cognitive functions were markedly recovered. These findings suggest that the novel collagen/heparan sulfate porous scaffold loaded with neural stem cells can improve neurological function in a rat model of traumatic brain injury. This study was approved by the Institutional Ethics Committee of Characteristic Medical Center of Chinese People’s Armed Police Force, China (approval No. 2017-0007.2) on February 10, 2019.

Key Words: collagen; heparan sulfate; injury; neural stem cells; regeneration; repair; scaffold; traumatic brain injury; morris water maze; motor evoked potential; synapses; myelin sheaths

Introduction

The treatment of traumatic brain injury (TBI) remains a major challenge all over the world because of the brain’s limited self-repair abilities after injury (Tam et al., 2014). Despite numerous efforts in recent years, no specific therapies can repair the injured site or reconnect injured neuronal circuits, likely because of complicated pathophysiological processes, cavity formation following brain tissue loss, and the poor

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self-repair capacity of nerve cells after TBI. Karlsson et al. (2000) suggested that neural stem cell (NSC) therapy is a safe and efficient method for replacing necrotic or damaged tissue after TBI. However, few studies have reported a clear therapeutic effect of NSCs delivered directly to the injury site after TBI. Transplanted exogenous NSCs are unable to effectively migrate to the local injury site because of a lack of extracellular matrix in this area, which results in very low NSC adhesion and proliferation rates (Cromer Berman et al., 2013). To enhance the targeted delivery of exogenous cells to the brain, biomaterial scaffolds loaded with stem cells have been widely studied as a potential treatment strategy to improve the ability of NSCs to promote recovery from neurological injury after TBI (Yan et al., 2019). However, an ideal biomaterial scaffold for TBI still needs to be developed.

Polysaccharides and proteins are promising natural polymers for the construction of biomaterial scaffolds, with the characteristics necessary for tissue engineering applications (Fischbach et al., 2009; Chen et al., 2012b). Previous studies have reported that polysaccharides can enhance stem cell expansion and differentiation via the fibroblasts growth factor receptor signaling pathway (Dombrowski et al., 2009; Smith et al., 2011). This signaling pathway plays an important role in mediating the dynamic process of stem cell differentiation into cells with neural lineages (Li et al., 2019b). Moreover, a variety of protein growth factors and chemokines can be combined with polysaccharides in vivo to prevent them from being affected by heat, acid, proteases, and other adverse factors, to allow their involvement in various metabolic roles by activating specific signaling pathways in living organisms (Murakami et al., 2015). Polysaccharides are therefore often used to prepare neural tissue-engineered scaffolds for repairing nerve tissue defects, and can protect neurotrophic factors from proteolytic degradation, prolong their half-life, and maintain a balanced internal environment (Murakami et al., 2015; Chen et al., 2017).

Chitosan and heparan sulfate are widely used polysaccharides in tissue engineering. Chitosan is limited in that chemical cross-linking agents, such as genipin or glutaraldehyde, are needed for the preparation of chitosan scaffolds. Because most of these chemical cross-linking agents have certain cytotoxicity and high brittleness, biological scaffolds made using this method potentially risk compressing peripheral nerves (Skop et al., 2013). In contrast, biological scaffolds prepared using heparan sulfate can be induced by physical cross-linking methods, such as ultraviolet irradiation, thus avoiding the aforementioned adverse reactions (Chen et al., 2017). Heparan sulfate is a polysaccharide that makes up the neuronal basement membrane and extracellular matrix, and plays an important role in nerve fiber regeneration (Zhao et al., 2015). Additionally, heparan sulfate is involved in stem cell proliferation and differentiation through its interactions with various proteins (Nurcombe and Cool, 2007).

Studies have demonstrated that, in the process of biomaterial scaffold fabrication, a crosslinking reaction between collagen and heparan sulfate can enhance the mechanical strength of the scaffold by increasing the β-sheet content (Lu et al., 2007; Chen et al., 2017). Natural protein biomaterials, such as silk fibroin, gelatin, and collagen, are commonly used to make biological scaffolds in the field of tissue engineering. Collagen is a naturally occurring protein that is widely used in tissue engineering (Hu and Zhang, 2019). It is a common component in the extracellular matrix, and provides an appropriate microenvironment for nerve cell proliferation, differentiation, and metabolism (Zhang et al., 2019a). Similar to silk fibroin, collagen possesses low immunogenicity and excellent biodegradability and biocompatibility (Gentleman et al., 2003; Simionescu et al., 2006). Furthermore, collagen has a pivotal role in providing essential signals that influence cell activity (Yannas et al., 2010). We therefore hypothesized that the biological activity of NSCs might be improved if NSCs were co-cultured with collagen. However, as well as excellent biological properties, an ideal biomaterial scaffold should also have qualified mechanical properties, and collagen has poor physical properties and thermal stability (Cornwell et al., 2007). Some measures have consequently been suggested to make up the defects of collagen, for example, the cross-linking of collagen with heparan sulfate. In the present study, we focused mainly on the role of a collagen/heparan sulfate (C/H) scaffold loaded with NSCs in the repair of TBI-induced neurological deficits in rats.

Materials and Methods

Animals

To obtain the NSCs, two specific-pathogen-free Sprague-Dawley rats at 15 days of gestation were provided by the Academy of Military Medical Sciences, China [License No. SCXK (Jun) 2019-0006]. Furthermore, 48 male Sprague-Dawley rats (aged 12 weeks, weighing 200 ± 10 g) were included in the in vivo study. These animals were provided by the Academy of Military Medical Sciences, China [License No. SCXK (Jun) 2019-0010] and were kept in specific-pathogen-free conditions at the Animal Center of Tianjin Key Laboratory of Neurotrauma Repair. All animal research protocols and welfare were carried out in accordance with the Guide for the Care and Use of Animals of the Institutional Ethics Committee of Characteristic Medical Center of Chinese People’s Armed Police Force, China (approval No. 2017-0007.2, approved on February 10, 2019). All experiments were designed and reported according to the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines.

NSC cultures and identification

Two pregnant rats were sacrificed with intraperitoneal injections of pentobarbital sodium (100 mg/kg; Solarbio Science & Technology Co., Ltd., Beijing, China). The bilateral cerebral cortices of the embryonic rats were immediately acquired under sterile conditions. The tissue was minced using microscissors and filtered through a 40 µm-sized mesh. A cell suspension was then prepared and primary NSCs were cultured as previously reported (Gao et al., 2015). The dissociated cells were seeded in T25 culture flasks (Corning Inc., Corning, NY, USA) at a density of 5 × 10^5 cells/cm^2 and cultured in growth medium, including serum-free Dulbecco’s modified Eagle medium/F12 (Thermo Fisher Scientific, Waltham, MA, USA), 4 M glutamine (Thermo Fisher Scientific), 20 ng/mL basic fibroblast growth factor (Protein tech Int., Chicago, IL, USA), 20 ng/mL epidermal growth factor (Protein tech Int., Inc.), 2% B27 (Thermo Fisher Scientific) and 100 U/mL penicillin/streptomycin (Bioss Co. Ltd., Beijing, China), maintained at 37°C in a 5% CO₂ humidified incubator (XY-Bioscience, Shanghai, China). The dissociated cells gradually formed neurospheres after 1–3 days of culture in the growth medium. Sediment was discarded and the floating neurospheres were collected by centrifugation at 1466 × g at 4°C for 5 minutes. The neurospheres were then seeded into a new T25 culture flask with fresh growth medium. To acquire purified NSCs, the aforementioned passage process was repeated. When the NSCs were sub-cultured to the third generation, NSCs were identified by their morphological features under a light microscope (Leica Microsystems, Frankfurt, Germany) and with nestin [a marker of NSCs (Jiang et al., 2020)] immunofluorescence.

C/H scaffold preparation

Collagen was obtained from fresh bovine tendon using the acid swelling/pepsin digestion method (Shreiber et al., 2003). Briefly, fat and fascia were trimmed from the tendon and ground using a blender (Miliab, Hangzhou, Zhejiang, China). Subsequently, the residues were precipitated in 0.05 M Tris buffer solution for 24 hours. An acetic acid solution and...
pepsin were then added to the solution, and the supernatant was collected. Next, 3.5 M NaCl solution was added to the supernatant to salt out the precipitation, followed by dialysis with deionized water at 4°C for 5 days. A previous study (Chen et al., 2017) demonstrated that a weight ratio of 20:1 (C/H) gives the best biocompatibility and mechanical properties for C/H scaffolds. The collagen and heparan sulfate (Nanjing Sai Hong Rui Biotechnology Co. Ltd., Nanjing, China) were therefore dissolved in 0.05 M acetic acid solution at this weight ratio and uniformly mixed using a magnetic stirrer (Wiggers, Berlin, Germany). The mixed solution was irradiated for 10 minutes with an ultraviolet lamp (365 nm, 18 W/cm²) to induce the cross-linking reaction. The mixture was then freeze-dried with a vacuum freeze dryer for 2 days and immersed in 1% NaOH solution for 12 hours. Finally, the compound C/H material was repeatedly washed in deionized water. The samples were then cut into 2 × 2 × 2 mm³ cylinders and sterilized using 60Co (radiation dose 15 kGy).

A portion of the C/H scaffold (1 cm³) was placed into the wells of a 12-well plate. For cell seeding, 100 µL of NSC suspension (1 × 10⁷ cells/mL) was seeded onto the scaffolds, and the C/H scaffolds loaded with NSCs were incubated for 7 days at 37°C in a 95% air and 5% CO₂ humidified incubator. The medium was refreshed every 2 days. The C/H scaffolds co-cultured with NSCs were denoted as the C/H + NSCs group, and the NSCs cultured alone were denoted as the NSCs group.

Mechanical properties of the C/H scaffolds

Differential scanning calorimetry (DSC) measurements

DSC measurements were performed as previously described (Chen et al., 2012a). Approximately 10 mg C/H + NSC samples were heated to 400°C at a heating rate of 1°C/minute in an argon atmosphere. A DSC 1 calorimeter (Mettler Toledo, Zurich, Switzerland) was used to measure the phase transition temperature of the samples during heating.

X-ray diffraction measurements

The crystallization behavior of C/H + NSC samples was measured using an X-ray diffractometer (Malvern Panalytical BV, Eindhoven, Netherlands) over the 2 theta range from 5° to 90° at a scanning rate of 5°/minute.

Fourier-transform infrared spectroscopic analysis

The C/H + NSC samples were analyzed using a Nicolet Nexus 870 infrared spectrometer (Thermo Fisher Scientific). The Fourier-transform infrared spectroscopic spectra were analyzed at a range of 4000–600 cm⁻¹, with a resolution of 4.0 cm⁻¹.

Porosity measurement

Dry scaffolds were degassed in anhydrous ethanol for 5 minutes until no bubbles escaped from the samples, and the volume of anhydrous ethanol was denoted as $V_a$. The volume of anhydrous ethanol was recorded before ($V_a$) and after ($V_b$) removing the wet scaffolds. The porosity of the scaffold was calculated according to the following formula (Zeng et al., 2015): porosity (%) = ($V_a - V_b$)/($V_a - V_b$) × 100.

Water absorption rate

The weight of the freeze-dried scaffold was recorded as $M_w$. The scaffold was then immersed in 0.01 M phosphate-buffered saline (PBS) at 37°C for 24 hours before being dried rapidly with filter paper. The weight ($M_a$) was then recorded. The water absorption rate of the scaffold was calculated according to the following formula (Zeng et al., 2015): water absorption rate = ($M_a - M_w$)/$M_w$ × 100.

Assessment of NSC morphology, viability, and proliferation

The adhesion and morphology of NSCs in the C/H + NSC samples were observed under a scanning electron microscope (SEM; Hitachi, Tokyo, Japan). Briefly, the C/H + NSC samples were successively fixed with 2% glutaraldehyde (Shandong Forte Disinfection Products Co. Ltd., Dezhou, Shandong, China) and 1% osmium tetroxide (Sigma-Aldrich, St. Louis, MO, USA). The samples were then dehydrated with a gradient of acetone, rapidly frozen to the critical point by liquid nitrogen, and freeze-dried for 12 hours. Finally, the C/H + NSC samples were coated with gold, and the adhesion and morphology of the NSCs were observed under the SEM. Simultaneously, different regions were randomly selected and the pore diameters of the C/H scaffold were measured under the SEM.

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich) at 1, 3, 5, and 7 days after seeding NSCs. For this experiment, 100 µL of NSC suspension (1 × 10⁷ cells/mL) was seeded to the C/H scaffold in 96-well plates and incubated at 37°C in a humidified incubator containing 5% CO₂. The medium was refreshed every 2 days. According to the manufacturer’s instructions, 30 µL CCK-8 was added to each well and stirred. The samples were then incubated for 4 hours at 37°C in a 5% CO₂ humidified incubator. Finally, the reaction solutions (300 µL) were transferred to a 96-well plate. The absorbance of each well at 450 nm was measured by a Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT, USA).

TBI model and scaffold transplantation

Forty-eight rats were randomly divided into four groups: sham ($n = 12$), TBI ($n = 12$), C/H ($n = 12$; C/H scaffold implantation after TBI), and C/H + NSCs ($n = 12$; C/H + NSC implantation after TBI). The rat model of TBI was prepared using the controlled cortical impact (CCI) method (cCCI Model 6.3; VCU, Richmond, VA, USA) as previously reported (Gao et al., 2015). Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and immobilized on a stereotactic frame. A 5 mm bony window was drilled in the right parietal region of the skull, 2 mm posterior to the coronal suture and 4 mm lateral to the sagittal suture. Moderate brain injury was induced with a 2 mm diameter cylinder in rats, with an impact velocity of 4.5 m/s, a depth of 2.0 mm, and a residence time of 120 ms. The rats in the sham group were not subjected to an impact.

For the C/H and C/H + NSCs groups, a 2 mm³ C/H scaffold or C/H + NSC sample, respectively, was transplanted into the cavity after TBI.

Morris water maze test

To evaluate spatial learning and memory, the morris water maze (MWM) test was performed from 31–37 days after injury, as previously reported (Yang et al., 2014). The MWM test included the spatial learning stage and the spatial memory stage. In the spatial learning stage, each rat (n = 8 per group) was released into a circular pool filled with a mixture of water and black ink (50 cm deep), and were trained to find a submerged platform and remain there for 2 seconds. If the rats failed to arrive at the platform within 60 seconds, they were provided with a stationary array of prominent maze cues and remained in the pool for 20 seconds. The spatial learning experiments were performed for 6 consecutive days, and the escape latency (the time taken to find the hidden platform) was recorded. The spatial memory experiment was performed on day 37, when the platform was removed from the maze. The quadrant dwell time (percentage of total time spent in quadrant IV) and platform crossings (the number of times the platform region was crossed) were recorded. Swimming patterns and parameters were collected and analyzed using an automatic tracking system (Ethovision 2.0, Noldus, Venlo, the Netherlands).

Motor evoked potential test

The motor evoked potential (MEP) test was performed at 2 months after injury to analyze differences in motor function between the left and right limbs of each rat as a...
result of damage to the cerebral motor cortex (Kim and Han, 2017; Jiang et al., 2018). First, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The recording electrodes were then inserted into the belly of the extensor carpi radialis of both forelimbs and the posterior tibial nerve of both hindlimbs. Next, the ground electrodes were inserted into the root of the tail, and the stimulating electrodes were located at the contralateral motor cortex, with the center taken as 2 mm posterior to the coronal suture and 4 mm lateral to the sagittal suture (Khatoun et al., 2019). The electrophysiology parameters were as follows: stimulation voltage 40 V, pulse width 0.2 ms, and stimulus frequency 1 Hz. The largest peak-to-peak amplitude and the earliest latency of MEP were recorded using evoked potential equipment (Nicolet; Thermo Fisher Scientific).

Magnetic resonance imaging
T2-weighted magnetic resonance imaging (MRI) was performed under general anesthesia at 1 month after injury to assess structural changes in the brain, using a 3.0 T MRI system (MAGNETOM Verio, Siemens, Berlin, Germany). T2-weighted images were obtained using the following sequence parameters: repetition time 3200 ms, echo time 83 ms, rapid imaging with refocused echoes (RARE) factor 10, field of view 19.2 mm × 19.2 mm, matrix size 320 × 320, number of acquisitions 20, and slice thickness 2 mm. Apparent diffusion coefficient (ADC) values were separately analyzed by two researchers using a double-blind method. The region of interest to be measured was located in the cortex, around the injury site. According to the results of brain tissue morphology displayed on T2-weighted images, three measurement points were randomly selected from the corresponding ADC images. The mean value across three measurement points was calculated and denoted as the ADC value of the region.

Neurological function observation
Modified neurological severity scores (mNSS) were used to assess neurological function changes in rats (n = 10 in each group) at 1, 5, 10, 15, 20, 25, 30, 35, 40, and 45 days after injury. The mNSS scores were graded on an 18-point scale that included reflex, motor, sensory, and balance tests. The detailed procedures were carried out according to a previous report (Wen et al., 2017). Briefly, each rat was trained and assessed before CCI to determine the normal score (0). The neurological scores were then calculated by blinded testers at different time points after CCI. A higher mNSS indicated more severe sensory and motor functional deficits.

Histology and immunohistochemistry
Animals were sacrificed at 2 months after CCI with an overdose of chloral hydrate and were then perfused with 200 mL normal saline and 200 mL 4% paraformaldehyde. For the histomorphological examinations, brain tissue was fixed with 4% paraformaldehyde for 24 hours and sliced into 5-µm thick sections on an automatic paraffin slice system (MEDIT, Hanover, Germany). Hematoxylin and eosin staining was performed to observe morphological changes and local tissue repair at the injury site. The cavity volume was measured to assess structural changes and local tissue repair. The cavity volume was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA) and computed using the following equations: n = 1 – 8 ∑ (A, + A, + 1) × d/2 (A = area, d = distance between sections) and % lesion volume = [(V, – V,)/ (V,)] × 100 (V, = ipsilateral (uninjured) hemisphere volume; V, = contralateral (injured) hemisphere volume).

Nissl staining and silver staining were also performed to analyze neuron and nerve fiber regeneration (Cheng et al., 2018). The silver staining method was used to detect new born nerve cells (Kee et al., 2002). Four rats from each group were intraperitoneally injected with BrdU (100 mg/kg; Cat# bs-0489R-1; Bios, Beijing, China), rinsed with PBS, incubated at 37°C for 1 hour with biotinylated sheep anti-rabbit IgG (1:200; Cat# PAB10842-E; Abnova, Wuhan, China), rinsed with PBS, and developed with 3,3′-diaminobenzidine. The slides were then stained with hematoxylin for 2–3 minutes, dehydrated, cleared, sealed, and differentiated with a hydrochloric acid/ethanol solution. Six random fields were visualized from each section under a DM IL inverted contrasting microscope (Leica Microsystems, Frankfurt, Germany). The numbers of BrdU-positive cells in each field of view were calculated for each group.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay
Brain tissue sections were prepared as previously described (Neher et al., 2014; Li et al., 2019a). Before staining, sections were dewaxed using a Drift baking machine (Yu De Co., Guangzhou, Guangdong Province, China) and dehydrated using graded ethanol. The apoptotic cells around the injury site were then detected using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) detection kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The 4′,6-diamidino-2-phenylindole (DAPI) was incubated in dark for 5 minutes to stain cell nucleus. Apoptotic neuronal cells were visualized under a fluorescence microscope (Leica Microsystems). Six fields were randomly selected on each slide for the quantification of TUNEL- and DAPI-stained cells by two researchers blinded to the experiment.

Transmission electron microscopy
Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) at 2 months after injury. The brains were rapidly isolated and the lesioned area was diced into 1 × 1 × 1 mm³ cubes on ice. Samples were fixed with 2% glutaraldehyde for 4 hours and washed with 1/15 M PBS for 15–30 minutes. The samples were then fixed with 1% osmium tetroxide for 2 hours and washed with 1/15 M PBS for 15–30 minutes. Next, the samples were dehydrated through graded ethanol and embedded in epon. An ultramicrotome (Leica UCT, Frankfurt, Germany) was used to make 70–90 nm-thick sections. These ultrathin sections were stained with uranyl acetate and lead acetate, and tissue ultrastructure was observed among the four rat groups using transmission electron microscopy (TEM). The synaptic curvature, cleft width, number of myelinated axons per 1000 µm², diameter of myelinated axons, and myelinated thickness were analyzed in each group.

Statistical analysis
The data are expressed as the mean ± standard deviation (SD). Statistical comparisons between groups were conducted using the two-sample t-test, or the one-way analysis of variance followed by the Student-Newman-Keuls test. A P-value < 0.05 indicated a statistically significant difference. All calculations were carried out using SPSS software (version 22.0; IBM, Armonk, NY, USA).

Results
Characterization of the C/H scaffold
Under the SEM, the pores of the C/H scaffold were evenly distributed and well interconnected (Figure 1A). The pore diameter was 265.4 ± 63.5 µm, with a range of 200–400 µm; pore diameters in this range are suitable for cell proliferation and adhesion (Bose et al., 2012). The NSCs were spherical (Figure 1B) and the expression of surface-bound nestin on
NSCs was determined using immunofluorescence (Figure 1C). Using light microscopy, NSCs were observed to be closely attached to the surface of the C/H scaffold. Some NSCs extended pseudopodia to contact each other, which even extended into the interior of pores (Figure 1D and E). Using SEM, NSCs were observed to gather together and were tightly attached to the C/H scaffold (Figure 1F). The CCK-8 results demonstrated that the C/H scaffold was not cytotoxic compared with the NSCs group. The results demonstrated that the C/H scaffold was not cytotoxic (P < 0.05; Figure 1H and I). In the Fourier-transform infrared spectroscopic analysis of the C/H materials (Figure 1J), the frequencies at 3294.78 cm⁻¹, 1632.43 cm⁻¹, 1547.77 cm⁻¹, and 1031.19 cm⁻¹ were correlated with the stretching vibrations of the C-H bond, C=O bond in COOH, NH2, double bonds, N-H bond, or C-N bond, respectively. In the X-ray diffraction analysis (Figure 1K), a sharp diffraction peak was observed at 21.82°, indicating that crystallization of the materials increased when heparan sulfate was added to collagen to generate a cross-linking reaction. The DSC image (Figure 1L) revealed that the heat flow of the C/H scaffold gradually changed with the constantly rising temperature. Three endothermic peaks appeared in the samples, at 113.95°C, 226.03°C, and 261.15°C. This result indicates that the samples begin to absorb thermal energy and decompose at these high temperatures.

C/H + NSC therapy enhances the recovery of neurological motor functions

The MWM test was carried out 1 month after injury to evaluate the influence of C/H + NSC transplantation on spatial cognitive function. In the spatial learning stage, the escape latencies in all groups were significantly reduced with training. In particular, C/H + NSC transplantation markedly promoted a decrease in escape latencies, while the escape latencies in the TBI group were the longest (Figure 2A). Student–Newman–Keuls test results revealed significant differences in escape latencies among the four groups at each time point (Figure 2C). The results of the MWM test indicated that the C/H + NSC group took a similar amount of time to reach the submerged platform as the sham group (P > 0.05), while the escape latencies were markedly lower in the C/H + NSC group compared with the TBI and C/H groups (C/H + NSCs vs. C/H, P < 0.05; C/H + NSCs vs. TBI, P < 0.01). In the spatial memory stage of the MWM, the quadrant dwell time and platform crossings were recorded. The number of platform crossings was higher and the quadrant dwell time was longer in the C/H + NSCs group compared with the TBI and C/H groups (Figure 2B). The Student–Newman–Keuls test demonstrated that quadrant dwell time and platform crossings were significantly higher in the C/H + NSCs group (C/H + NSCs group vs. C/H group, P < 0.05; C/H + NSCs group vs. TBI group, P < 0.01). The number of platform crossings was higher and the quadrant dwell time was longer in the C/H + NSCs group compared with the TBI and C/H groups (Figure 2B). The Student–Newman–Keuls test demonstrated that quadrant dwell time and platform crossings were significantly higher in the C/H + NSCs group (C/H + NSCs group vs. C/H group, P < 0.05; C/H + NSCs group vs. TBI group, P < 0.01). In the spatial memory stage of the MWM, the quadrants with the highest densities and the largest distribution changes were observed. Representative sections demonstrated that nerve fibers were evident in the perilesional region and injury site in the C/H + NSCs and C/H groups (Figure 3G and H). The C/H + NSCs group had the highest density and the largest distribution scope of nerve fibers in the perilesional region and injury site compared with the C/H and TBI groups (C/H + NSCs group vs. C/H group, P < 0.05; C/H + NSCs group vs. TBI group, P < 0.01), followed by the C/H group (C/H group vs. TBI group, P < 0.05). Additionally, the Nissl staining results (Figure 3I–M) showed Nissl body changes in the perilesional region and injury site in each group. There were larger numbers of Nissl bodies in the perilesional region and injury site in the C/H + NSCs group than in the TBI and C/H groups (C/H + NSCs group vs. C/H group, P < 0.05; C/H + NSCs group vs. TBI group, P < 0.01). Furthermore, the C/H group had the lowest density and distribution scope in the perilesional region and injury site among the four groups (TBI group vs. C/H group, P < 0.05).
after TBI

C/H + NSC therapy improves the absorption of hematoma vs. improvements in intracranial conditions. The MRI T2-weighted images demonstrated that C/H + NSCs and C/H groups had more BrdU-positive cells than the C/H group (P < 0.05).

C/H + NSC therapy improves the absorption of hematoma after TBI

As shown in Figure 4, the numbers of BrdU-positive cells per field at the injury site were higher in the C/H and C/H + NSCs groups than in the TBI group (P < 0.05, vs. C/H group; P < 0.01, vs. C/H + NSCs group). Additionally, the C/H + NSCs group had more BrdU-positive cells than the C/H group (P < 0.05).

C/H + NSC therapy improves the absorption of hematoma after TBI

The MRI T2-weighted images demonstrated that C/H + NSC transplantation after CCI had positive therapeutic effects, enhancing hematoma absorption and promoting improvements in intracranial conditions (Figure 3N). The edema volume was noticeably smaller in the C/H + NSCs group than in the other three groups. The TBI group displayed marked subcortical hyperintensities, induced by trauma-associated hydrocephalus, 1 month after brain injury. In addition, compared with the sham group, ADC values were markedly higher in the other three groups (Figure 3P). The ADC values of the C/H + NSCs and C/H groups were significantly higher than those of the TBI group (TBI group vs. C/H group, P < 0.05; TBI group vs. C/H + NSCs group, P < 0.01). Moreover, the ADC values of the C/H + NSCs group were lower than those of the C/H group (C/H group vs. C/H + NSCs group, P < 0.05).

C/H + NSC therapy promotes the rehabilitation of injured synapses and myelin sheaths

Using TEM, a representative series of ultrastructural micrographs demonstrating synapses and myelin sheaths at the injury site is shown in Figure 5. The synapses in the sham group had distinct presynaptic membranes, postsynaptic...
membranes, synaptic clefts, and synaptic interface curvature (Figure 5E). There were many synaptic vesicles in the presynaptic elements, and many dense materials attached to postsynaptic elements. The synapses in the other three groups showed unclear ultrastructure, with fused presynaptic and postsynaptic membranes, heterogeneous postsynaptic densities, and different synaptic cleft widths (Figure 5F–H). Quantitatively, the ultrastructure of the synaptic interface curvature was significantly increased and the synaptic cleft was significantly decreased in the C/H + NSCs group compared with the TBI and C/H groups (C/H + NSCs group vs. C/H group, \( P < 0.05 \); C/H + NSCs group vs. TBI group, \( P < 0.01 \)) (Figure 5I and M). We also analyzed ultrastructural changes in myelin sheaths among the four groups (Figure 5A–D). Compared with the C/H and TBI groups, the myelinated axon diameter, myelinated thickness, and number of myelinated axons were significantly increased in the C/H + NSCs group (C/H + NSCs group vs. C/H group, \( P < 0.05 \); C/H + NSCs group vs. TBI group, \( P < 0.01 \)) (Figure 5J–M). These results suggest that C/H + NSC transplantation after TBI promotes the rehabilitation of injured myelin sheaths and synapses.

TUNEL staining was performed at 2 months after brain injury to detect nerve cell survival and apoptosis (Figure 6). The numbers of TUNEL-positive cells at the injury site were significantly lower in the C/H and C/H + NSCs groups compared with the TBI group (\( P < 0.05 \); vs. TBI group, \( P < 0.01 \)). Moreover, the C/H + NSCs group contained fewer TUNEL-positive cells than the C/H group (\( P < 0.05 \)). These results demonstrate that C/H + NSCs transplantation after TBI prevents apoptosis of nerve cells.
Discussion

In the field of biomedical engineering, the regeneration and restoration of nerve tissue can be helped considerably by the transplantation of biomaterial scaffolds loaded with stem cells. A composite scaffold made of biodegradable materials can integrate cells into the central nervous system and brain with defined cellular density, thus delivering stem cells to the injury site to achieve excellent therapeutic effects (Carlson et al., 2016; Jin et al., 2016; Chen et al., 2018).

NSCs have been widely used in the treatment of TBI for their anti-hypoxia, anti-ischemia, and differentiation abilities, which can protect brain tissue from secondary injury and improve cerebral function after TBI (Pang et al., 2017; Clervius et al., 2019; Lee et al., 2019). How to better apply NSC therapies in the clinic is a difficult problem that needs to be addressed. Cell therapy is usually accomplished by smearing cell suspensions alone or as a local injection; however, therapy given this way can induce uneven cell distribution (such as insular deposition), cell necrosis caused by regional ischemia/hypoxia, and cell loss, which leads to poor therapeutic effects (Parr et al., 2008; Mothe et al., 2013). It is therefore important to choose an appropriate cell carrier as an extracellular matrix, for NSC proliferation and differentiation. A previous study has confirmed that a polymer scaffold can promote nerve regeneration through physical connection and chemical induction in rat models of TBI (Leung et al., 2012). In the present study, we designed a similar biomaterial scaffold and investigated the effects of this scaffold loaded with NSCs on nerve regeneration and restoration after TBI in rats. The CCK-8 assay revealed that the C/H scaffold had low immunogenicity and reliable biocompatibility, indicating that it is suitable for transplantation in vivo. Furthermore, in vitro tests demonstrated that the C/H scaffolds had suitable
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pore size and shape, porosity, and pore connectivity, which is conducive to the exchange of nutrients and metabolites for the growth of NSCs, and is also helpful to induce the seeding and proliferation of NSCs. The results of Fourier-transform infrared spectroscopic analysis indicated that the C/H scaffold was composed of various water- and lipid-soluble chemical bonds, thus providing an ideal microenvironment for cell adhesion and growth. The X-ray diffraction analysis showed that the composite scaffold had good crystallinity, which is beneficial to control its biodegradability and improve its mechanical properties. Excellent mechanical properties of biological scaffolds are conducive to NSCs (which are seeded on the scaffold) remaining at the injury site long-term without loss, thus better filling the lesion cavity, regulating the local microenvironment, and mediating the directional growth of newborn nervous tissue (Ogle et al., 2016).

The recovery of neurological motor function is a key indicator for evaluating therapeutic effects after TBI, and is the ultimate goal of clinical treatment. As expected, compared with treatment with C/H scaffolds alone, the C/H + NSCs group had better recovery in locomotor function and in the MEP and MWM tests. These findings suggest that NSC participation can further improve pathophysiological processes after TBI in vivo and provide an effective molecular delivery platform for the regeneration of nervous tissue. Nissl bodies are polymers composed of many regularly arranged rough endoplasmic reticulum, free protein bodies, and polynuclear bodies, and are the main place in which neurons synthesize proteins (Li et al., 2009). Our Nissl staining results also indicated a clear trend of neuronal regeneration at the injury site in the C/H + NSCs group. This result might be attributed to the suitable porosity and pore size of the C/H scaffold, which may provide an ideal microenvironment for NSCs to differentiate into neurons. Composite scaffolds not only provide a mechanically efficient support structure, but also induce neurovascular regeneration in targeted tissue by providing biological cues. Ideal composite scaffolds should have a mechanically efficient support structure that is suitable for newborn tissue growth, and should also have the ability to provide biological cues to induce tissue regeneration. In parallel to the in vivo experiments, TUNEL staining and TEM observation revealed that C/H + NSC transplantation after TBI promoted neuronal survival, synaptic remodeling, and neuronal axon regeneration. Immunohistochemical staining demonstrated that the number of BrdU-positive cells in the injury site was significantly increased in the C/H + NSCs group compared with the C/H group, indicating that C/H + NSCs therapy can provide an optimal neural circuit repair microenvironment by activating newborn nerve cells. Thus, the neuroprotective effect of C/H + NSC therapy may be superior to that of the C/H scaffold alone. The main reason for this finding is that the C/H scaffold + NSCs can directionally deliver NSCs to the injury site and promote their proliferation in the lesion area, thus avoiding cell loss and inactivation because of a lack of tissue support.

The low regenerative ability of neurons and excessive astrocytic proliferation can lead to neuroinflammatory reactions after TBI, which in turn causes fibrous scarring that inhibits damaged nervous tissue repair. Luo et al. (2019) demonstrated that NSCs stop the inflammatory reaction process by inhibiting astrocyte activation around the damaged area of nervous tissue, thus improving the ability of nerve cells to differentiate into neurons and increasing the expression of growth factors and anti-apoptotic proteins in the host brain. Hayashi et al. (2004) reported that heparans play positive roles in anti-fibrosis and anti-inflammation by preventing interactions between fibroblast growth factors and heparan sulfate glycoproteins on the cell surface. Based on these findings, we hypothesize that the reason that the C/H + NSCs group showed better recovery of sensory and motor functions was closely related to the anti-fibrotic and anti-inflammatory abilities of NSCs and heparan sulfate.

There are some limitations in our study. First, we did not measure the mechanical properties of C/H scaffolds before sterilization. The mechanical properties of most natural biological materials are changed to different degrees after sterilization by 60Co irradiation, because 60Co irradiation induces the denaturation of chemical bonds (Liu et al., 2013). However, the irradiation dose of 60Co in our study was 15 kGy, which is within the safe range (15–25 kGy). Irradiation doses in this range kill viruses or pathogens, but have minimal effects on the biological activity and physicochemical properties of the biomaterials themselves (Li et al., 2007; Wang et al., 2018; Xing et al., 2019). The key points of our study were the processes of C/H scaffolds co-cultured with NSCs and the transplantation of scaffolds into the brain after TBI; these processes were all based on the use of sterilized C/H scaffolds and had little to do with non-sterilized C/H scaffolds. Thus, we initially believed that the mechanical properties of C/H scaffolds before sterilization were irrelevant to the research results, and we did not measure the mechanical properties of C/H scaffolds before 60Co irradiation. Second, a key point in neural tissue engineering involves the ways in which the directional differentiation of NSCs can be regulated, and our research did not address this point well. Leipzig and Shoichet (2009) suggested that NSCs are more likely to differentiate into neurons on the surface of soft hydrogel than on stiff biomaterials, indicating that we may be able to adjust the C/H ratio in the C/H scaffold to find the most suitable ratio for NSCs to differentiate into neurons. This would further promote neurological repair after TBI. Another approach would be to regulate the directional differentiation of NSCs by adding the transcription factor ZEB2 (Deryckere et al., 2020) or gold nanocomposites (Zhang et al., 2020), which both have the ability to induce directional differentiation of stem cells. These aspects will continue to be the focus of future research. In summary, we can confirm that C/H + NSC therapy has the potential for application in neural tissue engineering. We hope to further develop different types of composite scaffolds, such as by loading neurotrophic factors or exosomes to the scaffolds, to provide greater feasibility for clinical application.

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