Preparation of Drug Sustained-Release Scaffold with De-Epithelized Human Amniotic Epithelial Cells and Thiolated Chitosan Nanocarriers and Its Repair Effect on Spinal Cord Injury

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1. Introduction

SCI is one of the primary causes of limb dysfunction [1]. For the past few years, the incidence of SCI has been on the rise, with dozens to tens of thousands of new patients with SCI every year around the world, mainly due to traffic accidents, high-altitude falls, violence, or sports injuries [2–4]. In the process of SCI, patients will have a series of local or systemic events such as hypotension, ischemia, and vasospasm [5]. These pathological changes can easily lead to organ dysfunction, shock, and even paralysis [6]. For now, SCI is usually treated conservatively in clinical practice, and drugs such as corticosteroids, gangliosides, scopolamine, and neurotrophy are commonly applied [7, 8]. Although it can relieve the SCI of patients, it is difficult to cure completely, and there are some toxic and side effects [9, 10]. With the increasingly serious threat of SCI, it is urgent to find an effective, stable, and rapid cure scheme for SCI in a clinic.

As a kind of stem cell, HAECs have been proven to be effective in transplantation therapy for central nervous system diseases [11, 12]. Moreover, previous studies have also revealed that HAECs have a very significant effect in the process of resisting cell aging and repairing cell damage [13, 14]. Compared with other transplanted cells, HAECs have higher safety and wider access, so it has great potential for clinical application [15]. However, the cell survival rate is extremely low through stem cell transplantation alone, which may not play an effective role in healing injury [16]. In recent years, it has been found in clinic that loading stem cells with biological scaffolds can effectively improve the...
survival rate and effect after stem cell transplantation, among which chitosan is a very commonly used loading material [17]. As a kind of natural macromolecular polysaccharide from shrimp and crab shells, chitosan is extremely mild and safe [18]. Chitosan can be electrostatically combined with nucleic acid to form nanoparticles, which not only have a very high protective effect on the DNA contained in them but can also make it release slowly and promote their binding to human cells [19]. Therefore, chitosan is applied as medicine or as a stem cell carrier for clinic medicine, and its effect has been generally recognized [20, 21]. According to related studies, sulphydrylation of chitosan can further enhance the cell permeability of nanoparticles, which is beneficial to cell binding [22]. Therefore, this research was devised to treat SCI by preparing de-epithelialized human amniotic basement membrane and using thiolated chitosan as nanocarriers, aiming to provide a reliable theoretical basis for future clinical practice.

2. Materials and Methods

Forty SPF SD rats, half male and half female, weighing 280–320 g, were from Zhuhai BesTest Bio-Tech Co., Ltd., with license number SYXX (Guangdong) 2020–0229. They were fed adaptively for one week. Rats were randomly assigned to 4 groups: transplanted HAECs (group A, GA), transplanted thiolated chitosan carrying HAECs (group B, GB), no processing (model group, MG), and control group (CG).

In GA, GB, and MG, rats were anesthetized with pento-barbitalum nutricum intraperitoneally and then fastened on the fixed mount. Next, the skin and muscle at T9-T10 level were carefully cut layer by layer, the neural plate at T9-T10 was excised, and the spinal cord was clamped with a microvascular clamp at a pressure of 20 g/cm² for 90 s, and then the incision was closed. In CG, a microvascular clamp was not applied to treat the spinal cord, and the others were the same as above-mentioned. A week later, rats in GA were transplanted with HAECs (ScienCell Research Laboratories, Inc.). The epithelial cell layer of HAECs was eliminated by trypsinase digestion, and 5 µL (2 cells/µL) cells were obtained and implanted into the transection site of model rats. In CG and MG, rats were implanted with the same amount of physiological saline. The chitosan (500 mg) was obtained and mingled with 8 mL of hydrochloric acid (1 mol/L). Then, the deionized water was supplemented to 50 mL, and then 1.38 g of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride was supplemented to continue. The pure thioglycollic acid (0.5 mL) was added and changed it to pH 5.0 with 2 mol/L sodium hydroxide. They were placed at ambient temperature for 6 hours in the dark, and then dialyzed with hydrochloric acid (5 mmol/L), hydrochloric acid (5 mmol/L), 1% sodium chloride, and hydrochloric acid (1 mmol/L), respectively, for 12 hours each time. Freeze-drying was applied to prepare the thiolated chitosan solid. The thiolated chitosan (1.25 mg) was added to the 5-

5'-dithio-bis-nitrobenzoic acid solution (0.5 mg/mL) for 3 h. After that, it was centrifuged for 15 min (15116 × g, 4°C) to obtain the supernatant, and the OD value (412 nm) was tested via an enzyme-labeling instrument. The morphology of thiolated chitosan was observed under a scanning electron microscope (SEM). HAECs and the prepared thiolated chitosan were cultivated for 24 hours, and then transplanted to rats in GB according to the scheme in 2.3. The concentration of HAECs was changed to 5 × 10⁷/hole and inoculated it into 96-hole plate. After 24 h, the culture medium was replaced, the thiolated chitosan was supplemented, and cultivated for 24 h, 48 h, and 72 h, respectively. Next, 20 µL MTT solution (5 mg/mL) was added. After 4 h, the supernatant was discarded and DMSO (150 µL) was supplemented. The OD value (570 nm) was tested via an enzyme-labeling instrument. The growth of HAECs cells with thiolated chitosan was compared with that without thiolated chitosan. In the three groups, rats were assessed by the Tarlov score [23] and the Basso Beattie Bresnahan (BBB) score [24] on the 0th, 3rd, 5th, and 7th days after transplantation. The higher the score, the better the situation.

After transplantation for 7 days, all the rats were killed by cutting their necks under anesthesia. The abdominal aorta blood (5 mL) was drawn and centrifuged for 10 min (1504 × g, 4°C) to obtain serum, which was put in a refrigerator at –80°C. In addition, the spinal cord tissue from the T9–T10 injured segment of rats was obtained and cut into small pieces of 1 mm² for a subsequent test. ELISA was used to test inflammatory factors such as interleukin-6 (IL-8) and interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α) in rat serum, and the kits were all from Invitrogen, the States. Spinal tissue samples were collected from samples in 2.9. The contents of malondialdehyde (MDA) and superoxide dismutase (SOD) in tissues were tested via xanthine oxidation and thiobarbituric acid. The kit was purchased from Beijing Biolab Technology Co., Ltd. and Sigma-Aldrich, the States. Spinal tissue samples were collected from samples in 2.9. They were fastened in 4% paraformaldehyde for 72 hours and prepared into paraffin sections (5 µm). Then, it was dyed in accordance with the specifications of the TUNEL apoptosis kit (Abcam, China) and counted under a microscope to calculate the apoptosis rate of nerve cells. Spinal cord tissue was put in RIPA lysate (Invitrogen, the States), and protein concentration was verified via BCA (Invitrogen, the States). SDS-PAGE electrophoresis was applied to transfer the film and seal it for 1 h. Beclin1 (1: 1,000), LC3 (1: 1,000), P62 (1: 1,000), and GAPDH primary antibody (1: 2,000) were supplemented overnight. Then, the film was washed with TBST and HRP-labeled goat anti-rabbit IgG secondary antibody was added (1: 4,000). ECL (Sigma-Aldrich, the States) was applied for detection, and the gray value was calculated.

All results were represented as (mean number ± standard deviation) and analyzed by SPSS19.0. Independent sample t-test was applied for comparison between groups. Univariate analysis of variance and LSD back testing were applied for comparison among groups. P < 0.05 was considered significant.

3. Results

3.1. Preparation of Thiolated Chitosan. According to the test, the sulphydryl group in 1 g of thiolated chitosan was approximately 214.52 ± 14.26, which was higher than in nonthiolated chitosan (26.42 ± 4.86) (P < 0.001,
3.2. Effect of Thiolated Chitosan on the Survival Rate of HAECs. MTT assay revealed that the HAECs curve with the intervention of thiolated chitosan was higher than that without intervention ($P < 0.05$, Figure 2).

3.3. Evaluation of SCI. On the 0th and 3rd day, Tarlov and BBB scores had no difference in GA, GB, and MG ($P > 0.05$, Tables 1 and 2), and they were lower than those in CG ($P < 0.05$, Tables 1 and 2). On the 5th and 7th days, the Tarlov and BBB scores in MG were the lowest among the three groups, followed by GA, and GB was higher than GA but lower than the CG ($P < 0.05$, Tables 1 and 2). In GA and GB, it increased from the third day ($P < 0.05$, Tables 1 and 2).

3.4. Comparison of Inflammatory Factors. In GA, IL-6, IL-10, and TNF-α were $(18.86 \pm 4.34 \text{ pg/mL})$, $(31.42 \pm 7.42 \text{ pg/mL})$, and $(52.62 \pm 9.57 \text{ pg/mL})$, respectively. In GB, they were $(11.87 \pm 3.42 \text{ pg/mL})$, $(22.42 \pm 6.72 \text{ pg/mL})$, and $(37.84 \pm 6.65 \text{ pg/mL})$, respectively. In MG, they were $(29.42 \pm 5.63 \text{ pg/mL})$, $(42.85 \pm 10.52 \text{ pg/mL})$, and $(84.63 \pm 11.74 \text{ pg/mL})$, respectively. In CG, they were $(9.62 \pm 2.05 \text{ pg/mL})$, $(12.84 \pm 4.26 \text{ pg/mL})$, and $(18.93 \pm 4.71 \text{ pg/mL})$, respectively. The levels of IL-6, IL-10, and TNF-α in MG were the highest, followed by GA. The levels of IL-10 and TNF-α in GB were lower than those in GA but higher than those in CG ($P < 0.05$, Figures 3(a)–3(c)). Nevertheless, there was no difference between GB and CG in IL-6 ($P > 0.05$, Figure 3(a)), which was lower than that in GA ($P < 0.05$, Figure 3(a)).

3.5. Comparison of Oxidative Stress. There was no difference in SOD between GB and CG ($P > 0.05$, Figure 4(a)), which was higher than the other two groups. But SOD in GA was higher than that in MG ($P < 0.05$, Figure 4(a)). The MDA in MG was the highest among the four groups, followed by GA, and the MDA in GB was lower than that in GA but higher than that in CG ($P < 0.05$, Figure 4(b)).

3.6. Comparison of Apoptosis Rate of Nerve Cells. The apoptosis rate of nerve cells was about $24.81 \pm 3.42\%$ in GA, $14.83 \pm 2.93\%$ in GB, $(42.82 \pm 5.63\%)$ in MG, and $(7.63 \pm 1.18\%)$ in CG. Compared with the four groups, the apoptosis rate of nerve cells in MG was the highest, followed by GA, and GB was lower than GA but higher than CG ($P < 0.05$, Figure 5).

3.7. Expression of Autophagy Protein. After detection, we concluded that the expression of Beclin 1, LC3, and P62 protein in the spinal cord tissue of MG was the highest among the four groups, while they were the lowest in CG ($P < 0.05$, Figures 6(a) and 6(b)). There was no difference in Beclin between GA and GB ($P > 0.05$, Figures 6(a) and 6(b)), but LC3 and P62 were higher than those in GB ($P < 0.05$, Figures 6(a) and 6(b)).

4. Discussion

SCI is one of the most common traumatic diseases at present, and its complications (such as muscle atrophy, infection, respiratory system disease) are becoming more and more complex and serious [25]. As one of the effective treatment schemes for SCI, stem cell transplantation has a significant effect, but it cannot be widely applied in clinical practice due to its low survival rate [26]. The emergence of nanodrug-carrying scaffolds may be the breakthrough of clinical application of stem cell transplantation in the future. Moreover, a large number of studies have revealed that oxidative damage, inflammatory response, and changes in cell biological behavior caused by oxygen free radicals all play a vital role in the development of SCI [27, 28]. Therefore, in order to confirm the effect of nanodrug-carrying scaffolds on stem cell transplantation in treating SCI, this research was devised to make a step-by-step analysis through the abovementioned points.

Firstly, we tested the sulphydryl groups in the prepared thiolated chitosan and found that the content of sulphydryl groups in the thiolated chitosan increased obviously. Previous studies have revealed that the sulphydryl groups can greatly destroy the intramolecular hydrogen bonds of chitosan, improve the water solubility of chitosan, and form disulfide bonds with the sulphydryl groups of mucin molecules, thus increasing the mucosal adsorption effect of nanoparticles and the absorption rate of encapsulated drugs in vivo [29]. Therefore, the thiolated chitosan is more convenient for drug loading than the nonsulphydryl chitosan, and it has a higher utilization value. By observing the cell survival of HAECS under the intervention of thiolated chitosan, we concluded that the growth of HAECS under the intervention of thiolated chitosan was obviously higher that than without intervention, which suggested that thiolated chitosan not only had no toxic and side effects on HAECS, but also could effectively enhance cell activation, so it had a crucial effect on making up for the low survival rate of stem cell transplantation. Therefore, we assessed the state of SCI in rats with SCI and control rats which were transplanted with HAECS, thiolated chitosan carrying HAECS, and did not intervene. The findings revealed that the scores of rats transplanted with HAECS and thiolated chitosan carrying HAECS in GA and GB were better than those of rats in MG, which also preliminarily revealed that the two transplantation schemes might have had definite improvement effects on SCI and the thiolated chitosan carrying HAECS was better. Then, we also concluded that the inflammatory factors in GB were obviously lower than those of rats in GA and MG by comparing the inflammatory factors of rats in the four groups, which revealed that the inflammatory reaction and inflammatory injury in the bodies of rats in GB were obviously ameliorated. However, the development of SCI is due to the massive release of inflammatory mediators,
Table 1: Tarlov score.

|     | GA     | GB     | MG     | CG     | F       | P      |
|-----|--------|--------|--------|--------|---------|--------|
| 0 d | 1.4±0.2| 1.5±0.3| 1.4±0.2| 5.0±0.0| 540.200 | <0.001 |
| 3 d | 2.0±0.2*| 2.1±0.2*| 1.9±0.3*| 5.0±0.0<sup>as</sup>| 401.700 | <0.001 |
| 5 d | 2.6±0.4<sup>a</sup>*| 3.1±0.3<sup>b</sup>*| 2.2±0.4<sup>as</sup> | 5.0±0.0<sup>as</sup>| 96.270 | <0.001 |
| 7 d | 3.2±0.5<sup>bg</sup>*| 4.2±0.4<sup>bg</sup>*| 2.3±0.5<sup>as</sup>| 5.0±0.0<sup>as</sup>| 74.100 | <0.001 |
| F   | 48.490 | 147.600| 12.100 | —      | 0.503   | —      |

Note. * represents the comparison with the same group on the 0th day, <sup>P</sup><0.05; # represents the comparison with the same group on the 3<sup>rd</sup> day, <sup>P</sup><0.05; @ represents the comparison with the same group on the 5<sup>th</sup> day, <sup>P</sup><0.05; & represents the comparison with GA at the same time, <sup>P</sup><0.05; $ represents the comparison with GB at the same time, <sup>P</sup><0.05; ^ represents the comparison with MG at the same time, <sup>P</sup><0.05.

Table 2: BBB score.

|     | GA     | GB     | MG     | CG     | F       | P      |
|-----|--------|--------|--------|--------|---------|--------|
| 0 d | 3.6±0.4| 3.8±0.3| 3.7±0.4| 15.8±0.5| 2219.000| <0.001 |
| 3 d | 6.2±0.6<sup>*</sup>*| 6.4±0.5<sup>as</sup>*| 6.1±0.6<sup>*</sup>*| 15.7±0.7<sup>as</sup>| 614.200 | <0.001 |
| 5 d | 8.4±0.5<sup>a</sup>*| 9.0±0.6<sup>as</sup>*| 6.4±0.6<sup>as</sup>| 15.9±0.6<sup>as</sup>| 514.400 | <0.001 |
| 7 d | 9.7±0.9<sup>b</sup> | 12.8±1.1<sup>as</sup> | 6.9±0.7<sup>as</sup> | 15.5±0.6<sup>as</sup> | 194.100 | <0.001 |
| F   | 181.000| 308.800| 59.050 | 0.799  | —       | —      |

Note. * represents the comparison with the same group on the 0th day, <sup>P</sup><0.05; # represents the comparison with the same group on the 3<sup>rd</sup> day, <sup>P</sup><0.05; @ represents the comparison with the same group on the 5<sup>th</sup> day, <sup>P</sup><0.05; & represents the comparison with GA at the same time, <sup>P</sup><0.05; $ represents the comparison with GB at the same time, <sup>P</sup><0.05; ^ represents the comparison with MG at the same time, <sup>P</sup><0.05.

Figure 1: Preparation of thiolated chitosan. (a) Sulphydryl content in thiolated chitosan and chitosan, ***P<0.001; (b) SEM image of thiolated chitosan.

Figure 2: Growth curve of HAECs. *P < 0.05.
Figure 3: Comparison of inflammatory factors. (a) IL-6 concentration; (b) IL-10 concentration; and (c) TNF-α concentration. * represents the comparison with GA, $P < 0.05$; # represents the comparison with GB, $P < 0.05$; @ represents the comparison with MG, $P < 0.05$.

Figure 4: Oxidative stress. (a) SOD concentration; (b) MDA concentration. * represents the comparison with GA, $P < 0.05$; # represents the comparison with GB, $P < 0.05$; @ represents the comparison with MG, $P < 0.05$.

Figure 5: Apoptosis rate of nerve cells. * represents the comparison with GA, $P < 0.05$; # represents the comparison with GB, $P < 0.05$; @ represents the comparison with MG, $P < 0.05$. 
resulting in the injury and necrosis of tissues and cells, thus aggravating the disease [30]. From this, we could conclude that HAECs carrying thiolated chitosan had a significant effect on the inhibition of inflammatory factors. The release of inflammatory factors is mainly activated by the peroxidation of tissues or cells [31]. The aggravation of oxidative stress is also an important pathological process that causes secondary injury to the injury site and its surrounding tissues after SCI [32]. After SCI, a large number of oxygen free radicals can be produced in tissues to cause oxidative damage to cells and damage neurons, glial cells, and other normal cells around them, resulting in cell necrosis and apoptosis [33, 34]. SOD and MDA are the most sensitive indicators of oxidative stress response, and their role in responding to oxidative stress in various diseases has been repeatedly verified [35, 36]. SOD has a strong ability to scavenge superoxide anion radicals, which indicates the antioxidation ability of the body. MDA is the final product of lipid peroxidation [37]. Our test results revealed that SOD levels in GB were raised while MDA levels declined, which indicated that oxidative stress reactions were obviously suppressed and the antioxidant capacity of tissues was improved. The detection results of TUNEL also revealed that the apoptosis rate of nerve cells in GB had obviously declined, which might be related to the effective inhibition of oxidative stress reaction. Finally, in order to further clarify the repair effect of thiolated chitosan carrying HAECs on SCI, we also tested autophagy proteins in spinal cord tissue. As an important factor causing cell injury and death, autophagy has been confirmed to increase obviously during SCI. In this test, we concluded that the autophagy proteins Beclin1, LC3, and P62 of rats in GB were obviously lower than those in MG. It could be seen that thiolated chitosan carrying HAECs also had an effective inhibitory effect on autophagy, and this inhibition might be the key to reduce oxidative stress response and inflammatory reactions of cells.

5. Conclusion

The nanodrug-carrying scaffolds of thiolated chitosan can ameliorate the survival rate of HAECs. The thiolated chitosan carrying HAECs may be applied to treat SCI by suppressing autophagy protein expression, oxidative stress response, and release of inflammatory factors in spinal cord tissue, which may be a new clinical therapy for SCI in the future.

However, we cannot understand exactly the therapeutic mechanism of thiolated chitosan carrying HAECs for SCI due to the limited conditions, and the improvement of thiolated chitosan for HAECs’ shortcomings may not only ameliorate the cell survival rate and the binding rate with the body, but also have a deeper and potential relationship between them, which is worth exploring and discovering. Nevertheless, the real clinical application of thiolated chitosan carrying HAECs needs to be confirmed by human experiments.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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