Survival and number of olfactory ensheathing cells transplanted in contused spinal cord of rats

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【Abstract】Objective: To observe the survival and the number of olfactory ensheathing cells (OECs) transplanted in the contused spinal cord, so as to provide a basis for further studying the biological action of OECs.

Methods: The rat spinal cords were contused with NYU-impactor II at T_{10} level by dropping a 10 g rod from a height of 25 mm. At the 1st week after injury, OECs isolated freshly from green fluorescence protein (GFP) of the rats were transplanted into the spinal cord at injured site and other two sites 1 mm apart from the caudal and rostral ends with the OECs number of 30000/µl × 3 =90000. The survival and the number of OECs were qualitatively and semi-quantitatively observed under the fluorescence microscope from 1 week to 13 weeks after transplantation. The motor function of the cord was evaluated with BBB score.

Results: GFP-OECs could survive at least for 13 weeks within the contused spinal cord. Their arrangement was from tight to loose and their number was decreased from 1 week to 13 weeks after injury. The average number of GFP-OECs was 536 at the 1st week, which was less than 1% of the number as compared with original transplantation. After then, the number of GFP-OECs was continually decreased, but the most obvious decrease was found during 1 week to 2 weeks. The extent of decrease at other time points was relatively mild. In contrast to the cell number, motor function of the cord was gradually recovered after transplantation.

Conclusions: The survival and the number of GFP-OECs are different between the animals and are affected by the pathological reaction of the host cord. Also it is related to the motor function recovery of the contused cord.

Key words: Spinal cord injuries; Transplantation; Olfactory ensheathing cells

Olfactory ensheathing cells (OECs) not only have the characteristics of the astrocyte, oligodentrocyte and Schwann cells, but also can secrete neuronotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glia cell line-derived neurotrophic factor (GDNF), neurotrophin 4/5 (NT-4/5), neureglin (NRG), etc and some substances such as L1, neural cell adhesion molecule, and laminin that are beneficial to nerve growth and regeneration. Therefore OECs are used to treat various kinds of acute or chronic animal spinal cord injuries (SCI) including transection and contusion as well as human spinal cord injuries. Huang et al recently reported the therapeutic effect of OECs transplantation in 656 cases of advanced spinal cord injuries, 457 cases of amyotrophic lateral sclerosis, 68 cases of cerebral palsy, 20 cases of multiple sclerosis, 11 cases of cerebrovascular sequelae, 10 cases of ataxia and so on, with 1 255 cases in total of nerve injuries and diseases. But the basic and clinical studies only focus on the recovery of the structure and function after spinal cord injuries, little attention is paid to the main body of treatment, e.g. changes of OECs after transplantation. So in order to provide basic evidence from the aspect of donor for OECs application in the treatment of SCI, we studied the survival and number of OECs within 13 weeks after implanting the green fluorescence protein.
(GFP)-OECs into the contused spinal cord of rats.

METHODS

Spinal cord contusion

The rats were raised and treated by the approval of the Animal Care and Ethics Committee of the Third Military Medical University, Chongqing, China. The SD adult rats weighing 350-400 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and incision was made after routine asepsis, the vertebral lamina of T10 spine were exposed, and then the spinal cord was contused with NYU-impactor II by dropping a 10 g rod from a height of 25 mm. Postoperative care included cleaning, nutrition, infection prevention and artificial urination.

Donor cell preparation

The GFP transgenic rats weighing 220 g around were deeply anaesthetized with pentobarbitone and killed by decapitation. The head was immersed into the 0.5% iodine solution quickly for 3 minutes and then immersed into 95% alcohol for deiodination. The skull was opened, olfactory nerves were cut, olfactory bulbs were picked out and their caudal parts were removed. The remaining head parts were immersed into the 4°C precooling D-Hanks solution (containing penicillin 100 U/L, streptomycin 100 µg/ml). Under a microscope, the piamater and its adhesive vessels were divided, the bulbs were opened along longitudinal axis and the internal white matter was removed. The remaining grey matter was washed with 4°C precooling D-Hanks solution for 3 times, then cut into 1 mm×1 mm×1 mm explants and digested with 0.125% trypsin for 20 minutes at 37°C. The digestion was ended with DFF20 (DMEM/F12/20%FCS) for 5 minutes. The explants were blown into unicellular suspension with a pipette. After placed for 5 minutes, the upper suspension was collected and inoculated into the poly-L-lysine (10 µg/ml) coated culture plate and cultured in an incubator at 37°C, 5%CO2 for 3 days. Following that, half volume of medium was exchanged with medium containing Forskolin (100 mg/L) and basic fibroblast growth factor (bFGF, 0.01 mg/L). The total volume of medium was exchanged every 2-3 days during the next 14 days.

NGFRp75, S-100 double immunofluorescent staining and Hoechst 33342 nuclear labeling were used for identification and purity detection of OECs (Figure 1). The OECs cultured for 10 days were used for immunofluorescence cytochemistry staining, and the method is briefly demonstrated as follows. The cultured OECs were fixed with 4% paraformaldehyde for 15 minutes for 2 times, incubated with normal goat serum for 30 minutes at room temperature, and placed into primary antibody working solution (Mouse anti-S-100, rabbit anti-nerve growth factor rector p75, 1:100) overnight at a 4°C refrigerator. Subsequently, OECs were incubated in secondary antibody working solution i.e. tetramethylrhodamine isothiocyanate labelled goat anti-rabbit IgG, fluorescein isothiocyanate (FITC) labelled goat anti-mouse IgG, 1:50, cultured for 1 hour away from light, then placed into 5 µg/ml Hoechst 33342 working solution cultured for 30 minutes away from light. The 0.001 mol/L phosphate buffer was used as substitution for primary antibody in control group. After drying and mounting, we used fluorescence microscope for observation at different emission.

The cell purity is represented with the average of the ratio of NGFRp75 and S-100 double positive cells number/Hoechst positive cells number. The OECs purity was over 95% in our experiment.

Donor cell transplantation

Seven days after spinal cord contusion, the contused spinal cords were exposed again. The brain solid positioner was used to fix the rats and 1 µl OECs suspension (30000/µl) was injected into the injury site and other two sites 1 mm apart from the caudal and rostral ends of the spinal cord with a glass microelectrode under a dissection microscope. The injection depth was 0.8 mm and the speed was 1 µl/min. The needle retained in situ for 2 minutes after injection.

The same method was used in the control group, but OECs were substituted with DMEM. The rats were cared and fed by the same protocol as experimental group upon awakening.

Histologic analysis

At 1, 2, 3, 4, 8, and 13 weeks after transplantation, the rats were anaesthetized and their thoracic cavities were opened. The rats were perfused with saline via the left ventricle until the effluent fluid was nearly colorless, and then perfused with 200 ml of 4% paraformaldehyde. The cords of 1 cm in length at the transplanted rostral and caudal sites were harvested and placed into the same stationary liquid to be fixed for 4 hours, and trans-
ferred into the 20% cane sugar solution overnight. The icy slicker (Leica CM 1900, Germany) was used to slice the spinal cords into 20 µm thick sections along the longitudinal axis of spinal cord. Sixty rats were used at the 6 time points with 10 rats in each including 5 rats in experimental group and 5 in control group.

Semi-quantitative analysis of donor cells
All the sequential sections were collected and observed with fluorescence microscope (Olympus BX50, Japan). GFP-OECs and autofluorescence host cells were distinguished by FITC and Rodamine excitation respectively. The sections containing the most OECs at each time point were selected and observed under microscope and continuous digital photos were shot with a colorized charge coupled device (CCD) digital camera (Olympus DP71, Japan) and at the software condition of 10× and 20×. The number of the cells was counted which represents the survival number of OECs at different time point after transplantation.

Motor function assessment
The extent of recovery of motor function in host rats was detected by the BBB scale with a double-blind and double-observer method. The scores from the two observers were averaged. The assessment began from the 1st week after transplantation and continued once a week till the 13th week.

RESULTS

Qualitative observation
The light green GFP-OECs were easily identified in host’s spinal cord. They were mainly distributed at the grey matter along the long axis, spread gradually to the rostral and caudal sites. In the early post-transplantation period (within 2 weeks), some OECs existed in the host’s spinal cord independently, and some interconnected as reticulate form. But because of the large number and the compact arrangement of OECs, the distribution of OECs often represented as conglomeration form under a low power microscope. Along with the migration, dispersion and decreasing amount of GFP-OECs, the independently existing GFP-OECs reduced gradually, and more OECs were interconnected by slender cell process so the reticulate form was more obvious (Figure 2).

Semi-quantitative observation
We counted the number of OECs under a microscope. One week after transplantation, the OECs’ number of each section averaged 536 ± 132.74. Compared with 90 000 during transplantation, the surviving number was less than 1% of original one. Two weeks after transplantation, the number decreased greatly (426 ± 124.14). Subsequently, the number was continually decreased but the declining rate was mild. Thirteen weeks after transplantation the number of each section remained at 321 ± 110.24. From Figure 2 we can see that the number is decreased as a declining curve form from the 1st week to the 13th week after transplantation, in which the curve is more steep during 1 week to 2 weeks, but statistical analysis suggests that there is no difference between adjacent groups. Compared the GFP-OECs number of another 5 time points with that of the 1st week time point, the statistically significant difference did not show till the 3rd week after transplantation (Figure 3).

Motor function assessment
The scale of the motor function in experimental group was 7.2 at the 1st week. The scale increased gradually and came to 17.5 at the 13th week as the rats’ surviving time extended, but could not reach the normal criterion. The motor function in control group recovered gradually as the rats’ surviving time extended but at a low level compared with experimental group. There was significant difference between the two groups at each time point (Figure 4).

DISCUSSION
The application of OECs to repair spinal cord injuries is a hot topic that is paid continuous attention and OECs are the only glia cells applied in clinic to date. Previous animal experiments indicate that OECs can promote the recovery of the structure and function of injured spinal cord. In clinical application, the results of phase I clinical trial abroad indicate that OECs is safe, feasible and without neuropathy aggravation. The domestic results of 656 cases of advanced SCI
treated with OECs show that OECs can improve the nerve function in advanced spinal cord injuries and retard aggravation. Although the theory and applied research of OECs has acquired certain achievements and the general situation is optimistic, the basic research is still insufficient and the clinical application is still controversial.

Spinal contusion is a classic spinal cord injury. How the survival status is after OECs transplanted into this kind of spinal cord injury is closely related to the effect of clinical application. Our results showed that the surviving number of OECs was less than one percent of transplanted number at the 1st week after transplantation, indicating that most OECs died. We suppose that the cause of death is related to the new environment created by donor cells and the death of donor cells caused by intense pathological reaction following SCI. In addition, the factitious handling during the process of detachment and purification of donor cells is another cause of cell death. These results suggest that the artificial impact should be avoided as far as possible, controlling pathological reaction and improving the local microenvironment should be paid more attention as well while utilizing OECs in treating SCI.

![Figure 1](image1.png)

**Figure 1.** Identification of OECs stained by Hoechst (A), P75 (B) and S100 (C). D was merged picture of A, B and C. ×200.

![Figure 2](image2.png)

**Figure 2.** Qualitative observation of the survival and number of OECs in contused spinal cord of rats from the 1st week to 13th week after transplantation. Their arrangement was from tight to loose and their number was decreased from 1 week to 13 weeks. A, B, C, D, E, F were GFP-OECs at the 1st, 2nd, 3rd, 4th, 8th, and 13th week after transplantation. Scale bar=100 µm.
How long OECs survive in host’s spinal cord is a very significant problem. We observed that the average number of GFP-OECs was 535 at the 1st week after transplantation and decreased to about 427 at the 2nd week. The average number decreased further but mildly during 3-13 weeks, suggesting that the pathological reaction is still intensive within 2 weeks after transplantation. After that the total number of donor cells maintained stable on the whole as the pathological reaction relieved. We also found that although OECs’ number did not change greatly in general, there were marked differences in the surviving number of OECs among individual rats. Especially at the 13th week, the mean number was 322 but the standard deviation reached up to 110, indicating that the effective cells number in rats after cell transplantation is different and so is the effectiveness of treatment.

Another interesting experimental phenomenon was that the function of injured spinal cord recovered gradually as the GFP-OECs number decreased after transplantation. It seemed that the cell transplantation was not related to spinal cord function recovery. Regarding the function of experimental group and control group, the former’s function recovery was better than the latter’s at each time point and there was a significant difference, indicating that OECs is the key factor impelling the function recovery of injured spinal cord. But the problem is why there is a significant difference in function recovery between experimental group and control group within 1 week after transplantation. We suppose that it may be relevant to the secretion of varied neurotrophic factors and the myelination function by OECs that promote the functional recovery or the temporary replacement of nerve function by some neurotrophic factors.

The relationship between the cell number and the spinal cord function recovery is another concerned problem. It affects not only the dose-effect relationship during practical usage but also the formulation of clinical principle and standardization on transplantation. Unfortunately previous studies concerning the problem are rare and our experimental results cannot give a definite answer. We have found that OECs number is few even absent in the rats whose motor function recovery is weak in the late stage of transplantation. This distinct experimental phenomenon, however, is hard to explain the dose-effect relationship between the cell number and function recovery. Therefore a large sample observation will be needed to give an exact answer in the future. In this experiment, the duration of observation is only 13 weeks after transplantation. It is our desire to observe 1 year or more in future studies so as to determine the end-result of OECs in injured spinal cord.

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REFERENCES


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