Original Article

Spinal Cord-derived Neural Precursor Cells as a Preventive Therapy for Spinal Cord Injury

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Abstract

Background: Spinal cord injury (SCI) as one of the most important diseases of central nervous system (CNS) without any definite treatment is still growing in incidence. In addition to trauma, some surgeries such as cardiac and thoracic aorta surgery may result in SCI as a complication. In last years, a promising approach has shed light on this CNS injury thanks to stem cell technology. Stem cell therapy could be considered as a good candidate for transplantation and enhancing neural regeneration in SCI. In this study, we identified the effects of spinal cord-derived neural precursor cells (NPCs) transplantation on SCI in after and before injury injection. **Materials and Methods:** NPCs were isolated from the adult rat spinal cord and cultured *in vitro* using complete culture media. After neurosphere formation, the cells were differentiated to neurons, oligodendrocytes, and astrocyte. The cells were transplanted to the rat model of SCI in 1 day before and 1 day after injury. The animals were followed for 12 weeks to assess their neurological performance. In addition, histological study and inflammatory cytokines levels have been studied. **Results:** Our results indicate that NPCs infusion both pre- and post-SCI could decrease the level of inflammatory cytokines. In addition, the neurological performance and histologic studies showed recovery after this type of injury using NPCs, and it might be due to inflammation modulatory effects on neural stem cells. **Conclusion:** NPCs therapy for SCI in both two-time points (before and after SCI) could be beneficial and make a neurological recovery. In other words, NPCs therapy could be considered as a therapeutic and also preventive approach for SCI.

Keywords: *Apoptosis, inflammation, neural precursor cells, protective, spinal cord injury*

Introduction

Spinal cord injury (SCI) is a serious neurologic problem with annual incidence of 15–40 cases per million worldwide,[1] and has devastating consequences including various degrees of neurological problems (such as loss of sensory and motor function, bowel and bladder dysfunction, spasticity, neuropathic pain, and autonomic dysreflexia), increased rates of cardiovascular problems, deep vein thrombosis, osteoporosis, and bed sores.[2-5] The combination of these problems can greatly impact one's functional ability and thus the general quality of life, emphasizing the need for developing treatment strategies for SCI. In some medical conditions, SCI could happen as a complication of thoracic aorta surgery and also cardiac surgery.[6,7] Almost 16% of the patients that undergo thoracic surgery may face with cord injury mostly due to ischemic phenomena.[8]

In the view of pathophysiology, two sets of events cause SCI; a primary damage to the spinal cord as a direct result of trauma, and a secondary injury due to initiation of reactive processes including inflammation, ischemia, lipid peroxidation, free radical production, demyelination, glial scar formation, and apoptosis and necrosis in the spinal cord tissue.[9] Various treatment strategies have been proposed regarding these pathophysiologic features. A group of studies have focused on neuroprotection by undertaking strategies to control and limit the mechanisms involved in secondary damage during the acute phase of injury.[10-12] In addition, axonal regeneration and replacement of lost neurons through growth-promoting factors and cell therapy are another approachs that have been widely considered.[13-19]

Different types of cells from various sources have been used for cell therapy purposes in SCI including stem/progenitor cells (embryonic stem cells, neural stem/progenitor cells, bone marrow mesenchymal stem cells, etc.), and nonstem cells (olfactory ensheathing cells, Schwann

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cells, etc.).[20] Stem cells have shown promising results in multiple studies performed on SCI models and are currently being tested in many laboratories.[13-17] Embryonic stem cells though, raise questions when it comes to the clinical application due to the ethical issues accompanied using them and also their tumorigenesis feature.[21,22] Furthermore, employing mesenchymal stem cells have some benefits by considering their potency and ability to migrate to the injury site.[23]

Neural precursor cells (NPCs) could generate the three major cell type: neurons, oligodendrocytes, and astrocytes; and their use also does not raise concern known with use of embryonic stem cells.[15] In addition, studies employing NPCs in primate, canine and rodent models of acute, subacute, and chronic SCI, resulted in the neurological promising outcome.[24-30] These cells are considered to have roles in both neuroprotection (through immune-modulation and angiogenesis mechanisms), axonal regeneration, and remyelination.[17] Taken together, NPCs have shown promise for cell therapy of SCI and many studies are considering the for clinical uses.

In this study, we have planned to identify the effects of systemic administration of neural precursor cell on SCI in two-time points. Neural stem cells therapy has good records to be a good candidate for treating spinal cord injuries; however, its usage before SCI has not been reported yet. We have decided to investigate if neural stem cells therapy could be an appropriate choice for preventive therapy of SCI in the conditions that may damage the spinal cord in some kinds of surgery.

Materials and Methods

All experiments were approved by Shiraz University of Medical Sciences Ethical Committee. All animals had free access to water and food during the experiment.

Experimental design

Sixty adult male Sprague-Dawley rats were selected and divided randomly into four groups (n = 15) as below:

Control group: Received no surgical intervention and no cell therapy

Sham group: Underwent SCI surgery

NPCs before SCI: Received 1000000 neural stem cells 1 day before SCI through tail vein

NPCs after SCI: Received 1000000 neural stem cells 1 day after SCI through tail vein

Neural precursor cell isolation, expansion, and characterization

Neural precursor cells were obtained from the adult rat spinal cord. Briefly, a 250 g adult male Sprague-Dawley rat was sacrificed, and the vertebral column was removed. The spinal cord was dissected and minced. Then, hyaluronidase (Sigma cat number: H1115000) (130 λ), trypsin (Gibco cat number: 25300054) (130 λ), and DNase I (Roch cat number: 04536282001) (25 λ) were added, the tissue was kept for 30 min in 37 ° C water bath with every 10 min shaking. For next step, the dissociated tissue was passed through 40 μm cell strainer, and then centrifuged for 5 min at 350 g. The isolated cells were transferred to T-25 cell culture flask with 5 ml complete neural precursor cells culture media containing DMEM/F12 (Gibco cat number: 10565018), 10 ng/ml bFGF (Sigma cat number: F3685), 20 ng/ml EGF (Sigma cat number: E9644), 2% B27 (Gibco cat number: 17504044), and 1% Pen/Strep (Gibco cat number: 15140122).

For differentiation of neural stem cells to tri-neural lineages cells, 5% fetal bovine serum (Gibco cat number: 26140079) was added to the culture media for 48 h. To detect neuron, astrocyte, and oligodendrocyte which were differentiated from neural precursor cells, immunostaining was done for microtubule-associated protein 2 (MAP-2), anti-glial fibrillary acidic protein (GFAP), and CNPase, respectively. For immunocytochemistry, the cells were fixed with paraformaldehyde 4% in + 4°C for 20 min. Following, permeabilization and blocking were performed with Triton 0.01% and goat serum 10%. After fixation, the cells were washed with phosphate-bufferred solution (PBS), and primary antibody for MAP-2 (Abcam ab32454, 1:500), GFAP (Dako Z0334, 1:1000), and CNPase (Abcam ab6319 1:500) were added, the cells were kept in room temperature for 2 h. Following incubation for primary antibody, the cells were washed with PBS, and the secondary antibodies were added and the cells incubated for 1 h in room temperature once again.

Spinal cord injury modeling

Compression model of SCI has been used in this study. Briefly, rats were anesthetized with halothane 2% and mixture of 1:1 N_2 and O_2. A midline incision was made from T5 to T9 vertebral column after using betadine as disinfectant. For reaching to spinal cord, the laminectomy was performed between T6 and T8, and spinal cord was compressed at the level of T7 by a 23 g aneurysm clip for 1 min. After compression, the wound was sutured and the rats received postoperation care.[31]

Basso, Beattie, and Bresnahan open-field locomotion scoring

For evaluation the motor performance of the rats, the Basso, Beattie, and Bresnahan (BBB) scoring was performed twice a week for 12 weeks by blinded examiner for each rat. The 22 BBB score (0–21) was used to assess the hindlimb locomotors recovery containing joint movement, stepping ability, trunk stability, and coordination. The score 21 represent no impairment which is in uninjured rats.[32]

Histology study

For evaluation necrosis and damaged area due to SCI, the cryosections of the damaged area were prepared and stained with H and E. The necrotic area was known due to existing some signs such as cells with swelling, pyknosis, and karyorrhexis nucleus, and disrupted cell membrane. For assessing the damage quantitatively, the sections were scored by blinded reviewer experts in this field from 0 to 3 by existing and intensity of inflammatory cell infiltration, neuronal vacuolation, and hemorrhage (0 is no evidence and 3 is sever).[33]

Apoptosis evaluation by assessment of caspase 3 activity

Activation of ICE-family proteases/caspases initiates apoptosis in cells. This assay is based on a spectrophotometric evaluation of chromophore p-nitroaniline (p-NA) after cleavage from labeled substrate (Asp-Glu-Val-Asp-Gly)DEVD-p-NA. The p-NA light emission could be measured using spectrophotometer at 400–405 nm. The activation of Caspase 3 was assessed 10 days after SCI. For this assessment, Caspase 3 assay kit from Abcam Company was used (Abcam Company, UK cat number: ab39401).

Enzyme-linked immunosorbent assay

Concentration of interleukin 1 (IL-1) β and IL-6 and in spinal cord (site of injury) were evaluated using IL-1 β (Abcam UK ab100768) and IL-6 (Abcam UK 100713). The levels of these proinflammatory cytokines were measured 5 days after SCI model.

Statistical analysis

All data reported as mean ± standard deviation in this study, and they were analyzed with one-way ANOVA test with Prism Graph pad 6.00. The statistically significant difference was set at $P < 0.05$.

Results

Neural precursor cells expansion, differentiation and characterization

The cultured neural precursor cells formed a sphere such as cell gathering which called neurosphere after 5 days. These isolated neural stem cells differentiated to three neural lineages by adding 5% fetal bovine serum to their culture media, they produce different cell types based on morphology feature. One of the most important characters for neural stem cells is capability of this cell type for differentiating to tri-lineages. They could differentiate to neurons, astrocytes, and oligodendrocytes which were stained with MAP-2, GFAP, and CNPase antibodies, respectively [Figure 1].

Locomotor function assessment by Basso, Beattie, and Bresnahan scores

The rats were examined twice a week by a blinded reviewer for 12 weeks to evaluate their motor function recovery. The neurological scores for sham group was (7.88 ± 0.91), for group that received cell therapy before SCI was (11.26 ± 2.70) and for group that received cell therapy after SCI was (11.27 ± 2.32). Both groups which received neural stem cells had better neurological

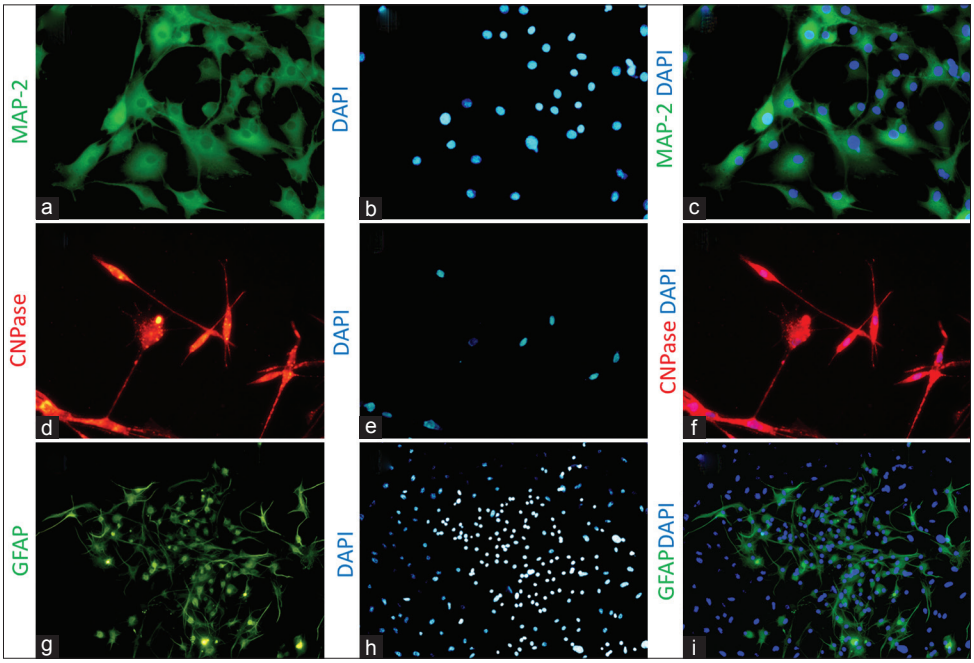

Figure 1: Differentiation of neural stem cells in three lineages. Differentiation of neural stem cells to neurons and immunocytochemistry with anti-microtubule-associated protein two antibodies (a-c). Neural stem cells could be differentiated to oligodendrocyte which is stained with the anti-CNPase antibody (d-f). Astrocyte differentiation of neural stem cells staining with anti-glial fibrillary acidic protein antibody

outcome and there was significant difference between them and sham group ($P < 0.05$). However, there was no significant difference between group that received neural stem cells before SCI and group which received neural stem cells after SCI. This data show that cell therapy by neural stem cells whether pre- and post-injury could enhance neurological outcome [Figure 2 and Table 1].

Histology study

The histological study has been performed based on the quantitative scoring as previously described. The histological score for sham group was (2.40 ± 0.21), for preinjury-treated group was (1.19 ± 0.27) and for postinjury-treated group was (1.83 ± 0.22). The quantitative score of histological study showed that both groups which received neural stem cells before and after SCI had lower histological damage in comparison with sham group ($P < 0.05$). Interestingly, the group that received NPCs before SCI showed better histological outcome in comparison with the group with postinjury cell therapy ($P < 0.05$).

This result indicates that neural progenitor cells transplantation could diminish *in situ* damage of the SCI, especially when the cells were injected before SCI [Figure 2 and Table 1].

Caspase 3 activity assessment for determining apoptosis

The apoptosis was evaluated 10 days after SCI by detecting activation of caspase 3.

The more caspase 3 activity represents more apoptosis. Absorbance at 405 nm, as an index for caspase 3 activity was (1.11 ± 0.07) in the sham group, (0.56 ± 0.04) in the group that received cell therapy before SCI and (0.77 ± 0.05) in the group that received postinjury cell therapy. The data indicate that all cell therapy groups have less apoptosis rate than sham group ($P < 0.05$). Furthermore, there is a significant difference ($P < 0.05$) between different time points of cell therapy, and the group with cell therapy before injury showed less activity of caspase 3 [Figure 3 and Table 1].

Inflammatory cytokines quantification

As described earlier, for exploring the underlying mechanism for preventive effects of the neural stem cells administration on the spinal cord, the level of two inflammatory cytokines (IL-1β and IL-6) were assessed. IL-1β level was (331 pg/ml ± 50.74) in sham group, (132 pg/ml ± 19.07) in the group that received cell therapy before SCI and (209 pg/ml ± 15.52) in group with postinjury cell therapy after injury. In addition, to IL-1β, the level of IL-6 another sign of inflammation was measured. IL-6 level was (203.66 pg/ml ± 8.02) in sham

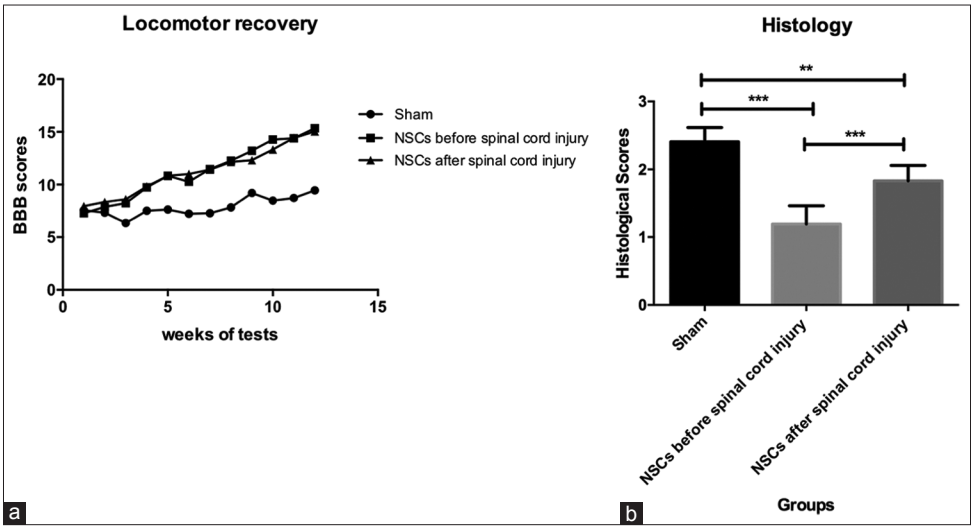

Figure 2: Neural examination during 12-week posttransplantation which shows functional improvement in groups with cell therapy treatment (a). Histologically, assessment of spinal cord tissue shows more recovery in cell therapy groups (b)

Table 1: Total characterization for different groups

	Control	Sham	Pre-SCI cell therapy	Post-SCI cell therapy
BBB score	-	7.88±0.91	11.26±2.70	11.27±2.32
Histological score	-	2.40±0.21	1.19±0.27	1.83±0.22
Caspase-3 activity	0.13±0.005	1.11±0.07	0.56±0.04	0.77±0.05
IL-1β (pg/ml)	88.33±11.23	331±50.74	132±19.07	209±15.52
IL-6 (pg/ml)	102.33±10.01	203.66±8.02	134.66±14.18	167.33±14.01

SCI – Spinal cord injury; BBB – Basso, Beattie, and Bresnahan; IL – Interleukin

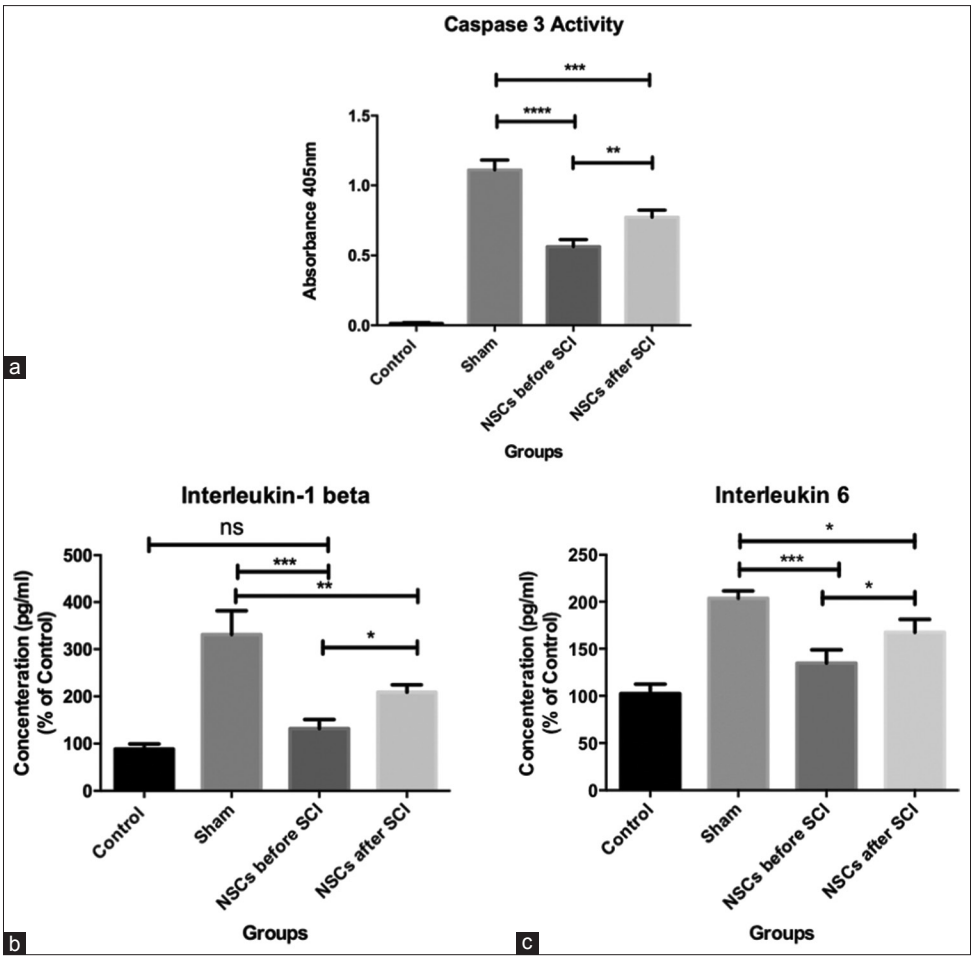

Figure 3: Caspase 3 activity quantification for apoptosis measurement that indicates lower rate of apoptosis in cell therapy received groups (a). The level of interleukin 1β and interleukin six measurements by ELISA method shows that they have been diminished in experimental groups (b and c)

group, (134.66 pg/ml ± 14.18) in group with preinjury cell therapy, and (167.33 pg/ml ± 14.01) in postinjury cell treated group. As the data show, theses inflammation indexes were diminished by neural stem cell therapy, and there is a significant difference between sham group and cell therapy groups ($P < 0.05$). In two cell treated groups, cell therapy before the injury had less amount of IL-1 ($P < 0.05$) [Figure 3 and Table 1].

Discussion

Neural Precursor Cells are able to produce three neural lineages in central nervous system including neurons, astrocytes, and oligodendrocyte. The rate of differentiation of NPCs to each of those cells is relatively dependent on their niches.[34] Due to informative reviews, the niche's effects on the differentiation of these cells could be due to the extracellular matrix, extracellular signaling, and also cell-cell interaction.[35-38] By considering the effects of the cells sources on differentiation and also their fate in this study, the spinal-cord-NPCs were used for SCI. In the view of clinic result, there are numerous studies which show that neural stem cells administration could help for recovering after SCI, and also the acute phase cell therapy could help achieving better outcome,[39] so we tested whether administration of neural precursor cells before SCI has some effects such as after SCI or not. By examining the animals during the experiment, all cell therapy groups had better improvement in comparison with a sham group which means the NPCs could help recovering after SCI in both pre- and post-injury time intervals such as previous studies. Although the most important index in the clinic is functional recovery, histological changes play a central role too. For evaluating the groups from histology aspect, the samples of each group received a score as described earlier. Both cell therapy groups had lower injury scores compared to the sham group, also neural stem cell therapy before SCI was as effective as postinjury transplantation or even more. Our data showed that transplantation of NPCs for SCI could diminish tissue injury such as previous studies.[40,41]

In our previous study, we found out transplantation of neural precursor cells for SCI could reduce apoptosis in SCI model,[42] also Hong *et al*. in 2014 showed that administration of neural stem cells may have anti-apoptotic effects in SCI model.[41] Following above, present study confirmed that NPCs might have anti-apoptotic effects on SCI model.

As far as researchers found about the primary and secondary damage of SCI, apoptosis plays a central part for both steps of SCI pathophysiology, and it is downstream of inflammation that caused by proinflammatory cytokines including IL1β and IL6.[43-46] For finding out the underlying mechanism of anti-apoptotic effects of neural stem cells in case of SCI, we evaluated the level of the two major pro-inflammatory cytokines IL1β and IL6. Our result shows that the administration of neural precursor cells in pre- and post-injury could attenuate these cytokines and this mechanism could be considered to justify the anti-apoptotic effects of neural stem cells. This possible mechanism exists in both group of our study, so we explore that the injection of NPCs before SCI could help more boosting the recovery than its administration after SCI.

Conclusion

SCI could be happened as a complication of some thoracic related surgery, so some patients have to take the risk of this kind of injury by undergoing the surgery of aneurysms of abdominal aorta or cardiac surgeries.[7,8] According to all above and by identifying the same mechanism of NPCs in pre- and post-injury injection, our study shows that this type of cell therapy (administration before injury) could be a safe and effective way to protect patients against this complication of unavoidable surgeries. Furthermore, this kind of cell therapy consideration could help clinicians prevent SCI in their surgeries. What we found new is, cell therapy by NPCs before SCI works in a same way that it works after SCI or even better in reducing some cytokines such as IL-6 and IL-1β and as a result, it could attenuate apoptosis after injury.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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