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Original Article

Neural stem cells transplantation alleviate the hyperalgesia of spinal cord injured (SCI) associated with down-regulation of BDNF

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Abstract: Transplantation of neural stem cells (NSCs) in the injured spinal cord has been shown to improve functional outcome. However, the influence of NSCs transplantation on the sensory function and analgesic behaviors has not been elucidated yet. Here, we investigated whether transplanted NSCs would improve sensory function in rats subjected to complete cord transection (T10) and explore the underlying mechanism. The rats were divided into sham, SCT (spinal cord transection), and NSC implanted groups. NSCs (3×10^6/ml) were implanted into injury site at the day after operation. Mechanical (the hind paw test) and thermal (the tail-flick test) were measured at 5 weeks. Immunohistochemistry and RT-PCR were used to demonstrate that expression of Brain-derived neurotrophic factor (BDNF) in the superficial of the dorsal horn. Consequently, the tail-flick latencies and paw withdrawal thresholds in NSC implanted group exhibit a significant higher than SCT group ($P < 0.05$). RT-PCR demonstrate that mRNA expression of BDNF was down-regulated remarkably in NSC engrafted rats. The present findings suggest that NSC transplantation inhibits neuropathic pain associated with BDNF down-regulation.

Keywords: NSC, BDNF, spinal cord transaction, sensory function, rat

Introduction

Spinal cord injury usually leads to serious neurological function deficits [29]. One of examples on these damages is paraplegia or pain, which has no effective method for the treatment of this phenomenon, although various treatments consisting of surgical, pharmacological and technological approaches have been involved in the affected patients [10, 31]. Recently, the approach of neural stem cells (NSCs) transplantation which featured hypotoxicity and high effectiveness may serve as a promising strategy for functional repair of spinal cord injury or nervous system injury diseases.

NSCs are multipotent cells which are capable of self-replication and differentiation into neurons, astrocytes or oligodendrocytes in the central nervous system [30]. NSCs have been isolated from adult brains striatum by Reynolds Since 1992 [27]. NSCs have the capacities to differentiate towards neurons and astrocytes in vitro, and it plays an important role in neural repair following injury [11]. Therefore, NSCs transplantation could be applied in human neural tissue injury and neurodegenerative disease treatment [3]. When the extensive bodies of evidences addressed the role of NSCs on the improvement of locomotor function, it kept to be investigated in the role of NSC for the sensory function regulation in rats with spinal cord transection. Also, the underlying mechanism for this phenomenon needs to be elusive. BDNF, as one of neurotrophic factors, has been noted in the development of neuropathic pain [6]. Incresaed BDNF in mice in spinal cord following peripheral nerve damage could result in hyperalgia [33]. Moreover, BDNF participated in regulating central sensitization and leaded to neuropathic pain. This process could be associated with activity of synaptic transmission of excitability neurons and weakness it of inhibitory neurons [2]. Recently, it has been found that exogenous BDNF through administration by cerebrospinal fluid (CSF) induced pain [7]. Previous study has reported that the NSCs transplantation resulted in thermal and mech-

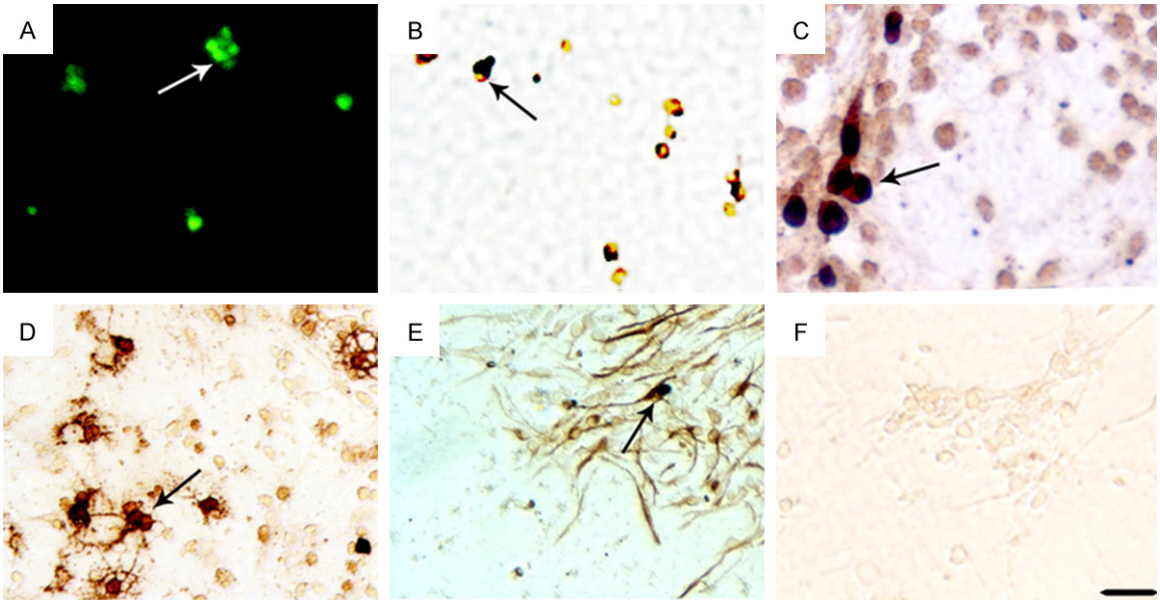

Figure 1. Morphology of NSCs and identification in vitro. Cultured Neurosphere at the 3th day was shown in (A) (green fluorecence), (B) (at the 7th day, nestin immunopositive, confirming undifferentiation). After passage with 10% FBS medium, Neurospheres exhibit NeuN (C), GFAP (D) or APC (E) immunopositive stainging, indicating their differentiation capacity. (F) was as negative control. Scale bar = 50 μm, showing in (F).

anical forelimb allodynia in spinal cord contusion rats [20, 22]. However it is not unclear under spinal and transaction in this study, we investigated continuously whether the NSCs transplantation could affect sensory function in rats with spinal cord transection, then to explore the possible underlying mechanisms for this phenomenon.

Material and methods

Animal and groups

Adult Sprague-Dawley rats (200~220 g) were supplied by the Animal Center of the Sichuan University. They were randomly divided into three groups (21 rats each group). Rats in Group A and B were subjected to complete spinal cord transection at the eighth thoracic vertebral level, corresponding to T10 cord. Then, Group B rats were subjected to NSCs transplantation. Rats in Group C were as sham operated. In each group, 5 animals were used for histological examination and immunohistochemical staining and 8 animals for RT-PCR analysis. The last 8 animals were used for behavior assessment. Treatment and care of animals was in accordance with the guidelines of the National Institute of Health Guide and Use of Laboratory Animals, USA. We tried our best to minimize animals suffering and reduce the number of animals used.

NSC cell culture in vitro: isolation, identification, and differentiation

NSC was dissociated from the hippocampal tissue of green fluorescent protein (GFP) transgenic embryonic mouse in our lab [32]. The protocol for NSC isolation and preparation in vitro has been described in previous study [32]. Briefly, hippocampal tissue were harvested and washed in D-Hanks' solution. After meninges of hippocampi were removed, the tissues were cut into several fragments, and then triturated to produce NSCs suspension. This was followed by a centrifugation and cell sedimentation was collected then cultured in a serum-free medium with DMEM/F12 (1:1, Gibco Laboratories, Grand Island, New York, USA), B27 (Gibco), and bFGF (20 ng/ml, Gibco). The cells at a density of 5×10^4-5×10^5 cells/ml were incubated in 24-well plate with 5% CO_2 at 37°C incubator. To identify the NSCs, part of NSCs were smeared then fixed on slides for 20 min by using 4% paraformaldehyde. After washing with PBS, NSCs were processed several steps by immunohistochemical SP two steps method by using cell-specific markers for undifferentiated stem cells (Nestin, 1:500, chemicon), for differentia-

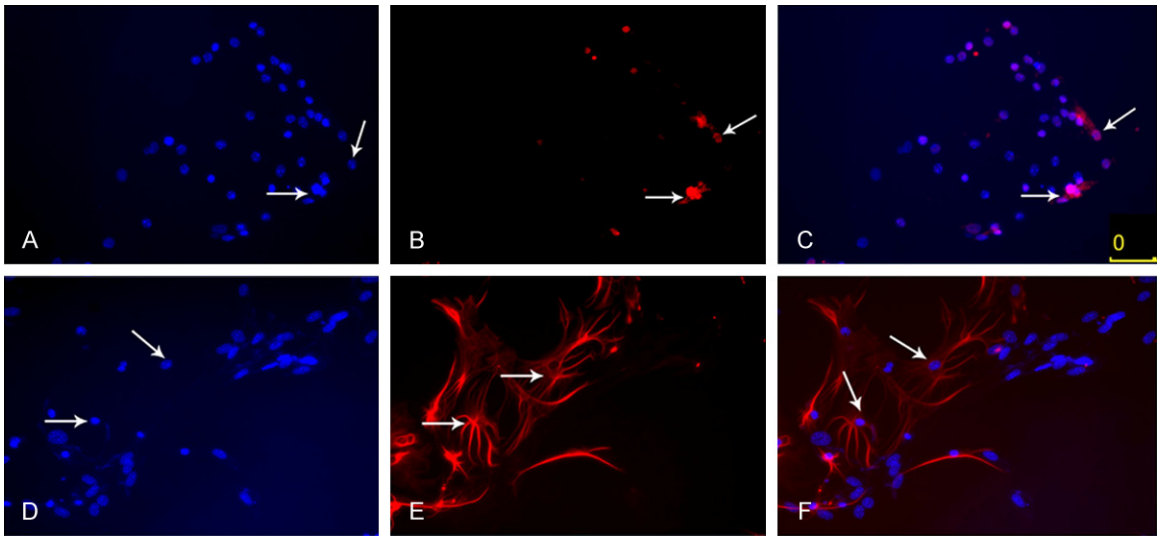

Figure 2. NSCs survival and differentiation in vivo. Transplanted NSC was marked by Hochest 3342, showing in (A, D), respectively. (A) few of them could differentiated into neurons (B) and astrocytes (E), indicating by Neun and GFAP immunofluorecent staining, respectively, showing in (B, E); (C and F) are a merged pictures from (A, B and D, E). bar = 50 μm, showing in (C).

tion on the detection of GFAP (chemicon, 1:200), APC (chemicon, 1:500) and NeuN (chemicon, 1:2,000). Lastly, the reaction was developed by PAB, as brown-yellow sedimentation. The cells were observed under microscope.

SCI model and NSCs transplantation

For the surgery, rats were anesthetized with an intraperitoneal injection of chloral hydrate (Beijing Chemical Reagents Company, China) at dose of 1 ml/100 g (3.6%). Then a laminectomy was carried out at the T8 vertebral level under surgical microscope and the spinal cord (T10) was transected completely. NSCs (3×10^6) were firstly performed Hochest 3342 labeled, then implanted into the injury site in the spinal cord. The sham-operated group underwent laminectomy without SCI. At last the skin wound was sutured and the rats were free access to food and water in isolated cages. Bladders of animals were emptied manually twice a day and penicillin was administrated once a day until bladder function recovered.

Sensory function assessment

Behavioral tests for thermal and mechanical allodynia were performed after SCI in tail and hindlimbs. Before the test, each animal was adapted to be handled.

Thermal stimuli (the tail-flick test): We used nociceptive stimulus by TAIL FLICK UNIT (UGO BASILE, Italy, 7360) to test the tail-flick latency, which is defined as the time between the onset of the heat stimulus and spontaneous tail withdrawal. The radiant automatically shuts off and records the latency when the rat moves its tail (temperature was 80°C, cut-off time was 15 sec).

Mechanical stimulus (the hind paw test): The rats were submitted to the paw pressure test by ANALGESY METER (UGO BASILE, Italy, 37215), according previous description of Randall and Selitto [26]. Nociceptive thresholds, expressed in grams, were measured by applying an increasing pressure to the hind paw until withdrawal (cut-off was 500 g).

Tissue processing for histology

Rats from each group were anesthetized i.p with 3.6% chloral hydrate at 5 weeks after transplantation. A left ventricle (LV) catheter was inserted and 200 ml normal sodium (NS) rapidly infused. After the outflow fluid became clear, 100 ml of 4°C 4% paraformaldehyde (0.1 mol/L PBS, pH 7.3) was perfused. Following perfusion, spinal cords were carefully dissected and fixed overnight in 4% paraformaldehyde. 20 μm thick longitudinal sections were obtained for immunofluorescence staining.

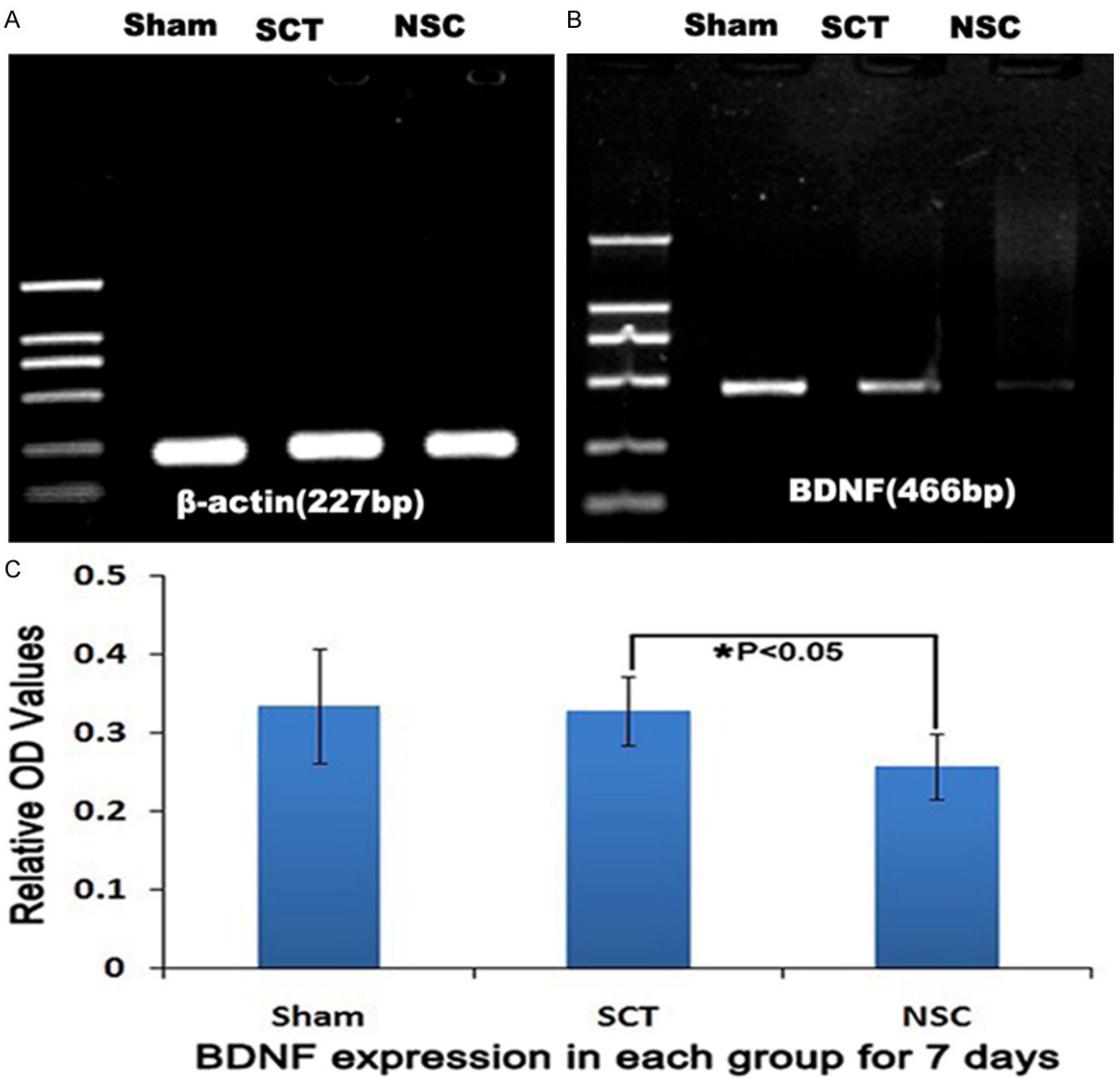

Figure 3. Changes on the BDNF mRNA in spinal dorsal horn. Electrophoresis band of β-actin and BDNF mRNA products resulted from RT-PCR was shown in (A and B), respectively. The quantitative analysis of BDNF was shown in (C), which demonstrated the BDNF was down-regulated significantly.

Immunofuorescent staining

For immunohistochemistry, sections were washed three times in 0.01 M PBS, 5 min each times, then incubated in 3% hydrogen peroxide at room temperature for 20 min to block the action of any endogenous peroxidase. This was followed by immersion for 30 min in PBS containing 0.3% Triton X-100 and 5% normal goat serum at 37°C, then processed for immunofluorescent demonstration of BDNF. Rabbit polyclonal BDNF antibody (1:100, Chemicon Company) was first used for the section incubation at 4°C overnight. After three times washing with PBS, Goat anti Rabbit Cy3 IgG was applied. To demonstrate the antibody specificity, PBS (0.01 M) was substituted for the primary antibody to as negative control. In addition, in order to determine whether or not the BDNF positive cells were neurons, we performed Neun immunofluorescent double labeling and 488 IgG immunofluorescent second antibody was used. Lastly, with an immunofluorescent microscope, the distribution and number of BDNF positive neurons in superficial dorsal horn was observed in the examined spinal cord segments. In order to the differentiation, transplanted NSC were labeled with Neun (1:100, Chemicon) and GFAP (1:100, Chemicon) by immunofuorescent staining same as above method.

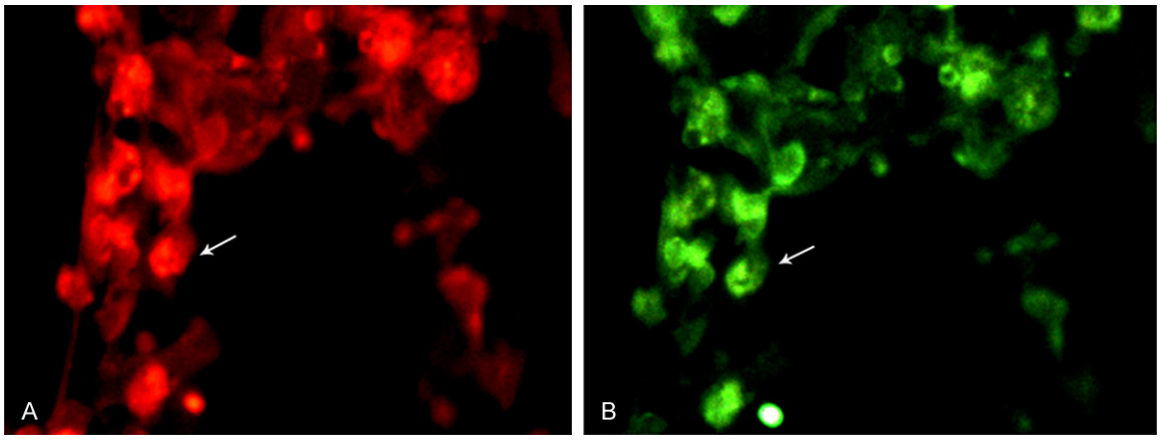

Figure 4. BDNF expression in superficial dorsal horn in vivo. BDNF positive cells was seen in spinal dorsal horn with emitting red fluorescence (A), which emits simultaneously green fluorescence, showing their neuronal marker. Bar = 50 μm, showing in (B).

RT-PCR

To demonstrate the change of BDNF mRNA, we harvested freshly and homogenized spinal tissues from each group. After the supernatant was harvested, the concentrations of RNA samples were measured with a Nanodrop spectrophotometer (ND-1000) (4 μg per experiment). RT-PCR was used to amplify the BDNF products, and β-actin was as an internal control. For RNA amplification, the first strand cDNA synthesis was prepared from 4 μg of total RNA, using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, USA). PCR was then carried out using the PCR MasterMix Kit (Fermentas, USA) for 30 cycles, consisting of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min. Gene primers were synthesized by TaKaRaCompany (Japan). The primer sequences for actin, BDNF, are as following. β-actin (227 bp): sense, 5'GTAAAGACCTCTATGCCAACA3', antisense, 5'GGACTCATCGTACTCCTGCT3'; BDNF (466bp): sense, 5'TCCCTGGCTGACACTTTT3', antisense, 5'ATTGGGTAGTTCGGCATT3'. RT-PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide, and visualized using an ultraviolet gel imager (Bio-Rad, USA). The optical density (OD) of each product band, including the objective gene and β-actin was obtained, and the OD ratio between BDNF and β-actin were calculated to semi quantify the objective gene level.

Statistics analysis

All statistical tests were evaluated by mean ± SD at a level of significance of 0.05. Data from this experiment was tested for statistical significance using the single factor analysis of variance with SPSS16.0 software.

Result

Identification and differentiation of NSC in vitro

In the 3-day-old primary culture, many small and rounded neurospheres with green fluorecence could be seen (**Figure 1A**). On the 7th day of culture, the number and diameter of neurospheres increased significantly, some of them comprised hundreds of cells. The neurospheres with nestin immunopositive staining were shown in **Figure 1B**, confirm their undifferentiation. After passage with 10% FBS medium, neurospheres exhibit NeuN positive or GFAP positive and APC immunopositive staining, indicating their differentiation capacity (**Figure 1C-F**)

Survival and differentiation of NSC in vivo

After implanted into the host spinal cord, NSC labeled by Hochest 3342 was seen around transection site (**Figure 2A**) A small percentage of the NSC expressed either neuronal (NeuN) (**Figure 2B**, **2C**) or glial markers (GFAP) (**Figure 2D-F**) at 4 weeks after transplantation.

Changes of BDNF mRNA in spinal cord following NSC transplantation

Following NSC transplantation, the mRNA expression for BDNF in spinal cord were detect-

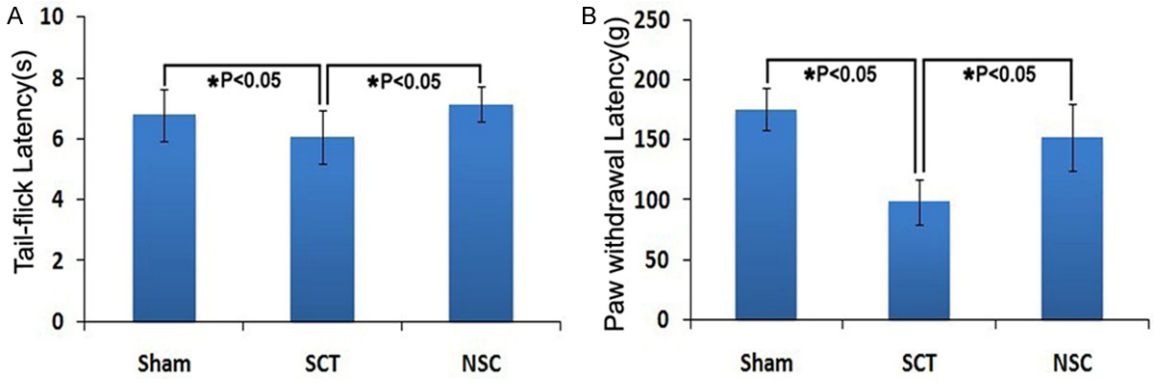

Figure 5. Evaluation of sensory function. SCT greatly decrease the latency of PWL in SCT group, and NSC grafts significantly reversed the latency of TFL, PWL in SCT group ($P < 0.05$), showing in (A and B).

ed in the primary culture of neurospheres and decreased significantly ($P < 0.05$) at 7 dpo when compared with the SCT group (**Figure 3**).

Localization of BDNF in the spinal cord

BDNF positive cells was mainly observed in gray matter, known as morphologically neurons. Especially, several BDNF positive neurons were found in superficial layer of dorsal horn in SCT group (**Figure 4A**). These BDNF positive cells could simultaneously labeled with Neun, a neuronal marker (**Figure 4B**). NSC transplantation results in a significant decrease in the number of BDNF positive neurons in superficial layer of dorsal horn in SCT group (SCT: 9.3 ± 1.6, NSC: 6.5 ± 0.9, $P < 0.05$).

Evaluation of sensory function

Thermal stimuli test showed that the tail flick latencies (TFL) in sham group were 6.17 ± 0.89 s. SCT did not alter the TFL (7.07 ± 0.74 s) when compared with sham one ($P > 0.05$). However, NSCs transplantation significantly delayed TFL (7.14 ± 0.57 s), and it exhibited a significant difference compared with SCT group ($P < 0.05$). The quantitative analysis was shown in **Figure 5A**.

Mechanical stimulis showed that SCT significantly decreased PWL (98.13 ± 19.05 g) compared with sham one 175.33 ± 17.67 g, while NSCs effectively increased PWL to 151.88 ± 27.86 g ($P < 0.05$) (**Figure 5B**).

Taken together, our data showed SCT could not induce thermal sensitization pain, but triggered mechanical pain. NSCs transplantation significantly improved mechanical pain.

Discussion

This study showed that transplanted NSC could result in alleviation of the hyperalgesia in injured spinal cord at T10 cord level after SCT, indicated by Mechanical and thermal test. This is first time to demonstrate that NSC transplantation in acute stage could relieve the neuropathic pain in rats with SCT, which is different from previous study that NSC implantation results in a hyperalgesia in contusion spinal cord.

NSCs are neurogenic and exist in the central nervous system (CNS). By exposure to different growth factors, NSCs can grow and maintain the capacity for self-renewal and generate differentiated progeny that can integrate functionally [4] and repair the damaged CNS [8, 13, 24, 25]. NSCs can also secrete neurotrophic growth factors and against excitotoxicity for the treatment of SCI [9, 18, 19]. Currently, NSC implantation into injured spinal cord could improve the motor function, which has been well documented [9]. Whereas, Melissa Y. recently reported that murine C17.2 NSCs resulted in thermal and mechanical forelimb allodynia (Melissa et al. 2006) in the contusion spinal cord. This indicated NSCs play a passive role in the sensory functional improvement in contusion cord. Therefore, it is worthy to investigate on the role of NSCs in transetion spinal cord injury. We found that NSCs can alleviate the hyperalgesia. The difference from the report of Melissa Y. in our study could be attributed to as following: a)

contusion injury fail to completely transect the nerve fibers tract, instead, inflammation is severe, whereas in SCT model, nerve fibers tracts have been completely transected, in later condition, neuronal injury is mainly resulted from axonal transetion. b) The anatomy pathway especially ascending conduct tract under contusion injury is integrated, but it encountered the inflammatory environment in the contusion spinal cord when sensory pathway ascended to brain. NSCs transplantation therefore may active the reaction of ascending nerve, which corresponds to the sensitization of sensory function lastly produce pain. Comparatively, under cord transection, the pain information could not be sent to brain but limit local spinal cord, it therefore may give a different reaction after NSC transplantation. Nowadays, there were a little report to demonstrate the improvement in sensory function and the underlying mechanisms in NSC treated SCT rats. Our finding is the first time to address that BDNF may be involved in a regulation of pain to improve sensory function in SCT rats after NSC transplantation.

BDNF, a member of the neurotrophic family, combined with its receptor, trkB, has been well identified (Pease et al. 2000; Perez et al. 1995). As a classic neurotrophic factor, several evidences showed that BDNF take part in the induction of neural repair. BDNF possesses autocrine and paracrine neurotrophic functions for varieties of neuronal populations. Injection of BDNF leads to stimulation of hindlimb activity after spinal contusion, and is associated with intensive growth of cholinergic fibers. In another report, grafting of BDNF-secreting fibroblasts in contusion injury resulted in advanced locomotor recovery similar to what we observed previously. However, recent studies showed BDNF is related to pain induction ([6, 7], Wang et al. 2012; Zhang et al. 2011). Therefore, the role of BDNF in injured spinal cord is complicated because it possesses both neuropathic [5] and neurotrophic functions [17].

As for as pain circuitry, primary sensory neurons can express BDNF [1], and colocalizes with Calcitonin Gene-Related Peptide (CGRP)-containing arborizations of afferent nociceptive C-fiber central terminals in the superficial dorsal horn [5]. In the present study, we found the BDNF positive neurons could be seen in superficial layer of the dorsal horn, indicated that the BDNF, expressed in post-synaptic neurous site, could involve in pain transduction. It has been reported that BDNF, as an endogenous manipulator, acted through reinforcement of spinal reflex responses (Kerr et al. 1999), and that contribute to hypersensitivity after peripheral inflammation [21]. In this study, we found that NSC transplantation give the result of alleviating the hyperalgesia in transected spinal cord, and BDNF has been down-regulated, as evidenced by RT-PCR. These suggested that BDNF could be as crucial candidates for relieving the pain in NSC grafting rats following cord transection.

Together, this study indicated that NSC implantation could improve sensory function and alleviate neuropathic pain in rats with SCT. The underlying mechanism may be correlated with the decrease of BDNF. These findings therefore amplify the usage of NSC transplantation for the treatment of SCT on the improvement of sensory function in future clinic trial.

Disclosure of conflict of interest

None.

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