



Induced Pluripotent Stem Cell- Derived Neuronal Precursors Ameliorate Sensory and Motor Ability in Non-Human Primates Suffering Spinal Cord Injury

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ABSTRACT

Background: The sensory and mobility failure associated with spinal cord injury (SCI) is desperately complicated due to the pathological events that occur sequentially in consequent to the injury.

Objectives: Herein, we applied neural stem cells, derived from human iPSCs (hiPSC-NSCs), to ameliorate the behavioral complications of contusive SCI in Rhesus monkeys, in sub-acute phase.

Methods: hiPSC-NSCs were maintained and characterized in vitro for general NSCs as well as hind-limb specific gene and protein expression prior to transplantation. Moreover, Masson's trichrome staining (MTS) in addition to luxol fast blue (LFB) were performed to determine the fibrotic scar reduction and myelination respectively. Tarlov's scale were utilized to score the motor improvement, plus, sensory perception evaluation throughout six months following the injury.

Results: hiPSC-NSCs were identified to own NSCs' exclusive properties in vitro by SOX2, DCX and NESTIN in addition to NESTIN, PAX6, SOX1, HOXA2 and HOXB2 protein and gene expression. Additionally, hiPSC-NSCs caused remarkable depletion in fibrotic scar and enhance myelination; spinal shock, sensory responses, reflexes and motor function were improved over six months.

Conclusions: Our findings suggest that hiPSC-NSCs lead to promising recovery after SCI, therefore, this source of NSCs provide a therapeutic potential in clinical studies.

Keywords: hiPSC-NSCs; spinal cord injury; Sub-acute phase; Rhesus monkey; Sensory perception; Motor activity.

INTRODUCTION

Traumatic SCI is described as physical damage to the spinal cord that causes temporary to permanent neurological and functional impairments, based on the lesion's severity (1). Clinicians have employed surgical, pharmacological and physical rehabilitation to overcome the SCI desperate side effects (2). Cell-replacement therapy has also been explored for potential uses such as modulating the inflammatory response, providing trophic factors, remyelination and neuronal regeneration (3). To date, numerous cell sources have been under discovery, while general neural stem cells, derived from various sources, are pronouncedly more likely to be beneficial in SCI treatment, even for clinical trials (4,5), beside animal models (6,7).

NSCs, regardless of their origin, are capable of releasing neurotrophic factors (8) in addition to compensating for neuronal (9) and oligodendrocyte loss (10) within the lesion; they are able to reduce oxidative stress (11), control inflammation (12), promote angiogenesis (13), decrease neuropathic pain (14) and restore sensory-motor behavior (15–18). Accordingly, NSCs, derived from diverse sources, suggest equal therapeutic potential for SCI treatment, whereas, iPSC-NSCs offer the additional autologous transplantation possibility, which requires further functional examination in non-human primates (19).

In the present study, we aim to exclusively address the physiological consequences of hiPSC-NSCs on sensory perception and motor activity using the Rhesus monkey. Indeed, the impact of hiPSC-NSCs has already been determined to be restorative in mouse model of SCI (18) while non-human primates were preferred here due to their profound analogy to human in spinal cord size, anatomy, and sensory/motor behavior (20) and more importantly, their equivalent immune reaction as well as physical deficit following the SCI (21). hiPSC-NSCs were transplanted at the sub-acute phase to avoid the deleterious consequences of severe primary inflammation, as accomplished by the injury (1). Since the *in vivo* fate of NSCs in injured primates has been indicated as tripotent (capable of differentiating into neuron,

astrocyte and oligodendrocyte) (10), we solely focused on the sensory/motor consequences of hiPSC-NSCs.

MATERIALS AND METHODS

hiPSC-NSCs *in vitro* maintenance

hiPSC-NSCs were obtained from the human iPSC lines, ROYAN hiPSC1 (passage 13) (ROYAN Stem Cell Bank, Tehran, Iran). hiPSC-NSCs *in vitro* maintenance and characterization of hiPSC-NSCs were thoroughly described in our previous study (22). The hiPSC-NSCs were cultivated on poly-L-ornithine (15mg/ml, Sigma-Aldrich, P4707, the USA) as well as Laminin (1 mg/ml, Sigma-Aldrich, L2020, the USA) substrate and maintained in Dulbecco's modified Eagle medium (DMEM)/ F12, supplemented with Knockout serum replacement (5%), N2 (1%), B27 (0.1%) (all from Gibco, USA), bFGF (40 ng/ml; ROYAN Biotech, Iran) and EGF (20 ng/ml; ROYAN Biotech, Iran) through a daily medium exchange routine.

Immunofluorescence staining

hiPSC-NSCs, were fixed by 4% paraformaldehyde (Sigma-Aldrich, 158127); permeabilized in the blocking solution containing 1% (w/v) of the secondary antibody's host serum together with 0.1% Triton. Primary (against NESTIN, SOX2, and DCX) and secondary antibodies (22) were applied, followed by 0.1 µg/ml DAPI (Sigma-Aldrich, D8417, USA) for nuclei staining. Further visualization and imaging were performed via an Olympus IX71 Fluorescence Microscope (Olympus, Tokyo, Japan) with a DP72 digital camera, and cell counting was performed in ImageJ.

RNA isolation and RT-PCR

Total mRNA (at P13-15) was extracted using RNase plus Universal Mini Kit (Qiagen, 73404) following by cDNA (2 µg) synthesis using a Revert Aid First-strand cDNA Synthesis Kit with random hexamer primer (Fermentas, k1632) according to the manufacturer's instructions. QRT-PCR was performed by a Power SYBR Green Master Mix (Applied Biosystems) via Rotor-Gene 6000 (Corbett Life Science) for selective genes (*NESTIN*, *SOX1*,

PAX6, *HOXA2*, *HOXB2*) (22). The comparative gene expression level in hiPSC-NSCs (compared to iPSCs), was normalized to GAPDH, using $\Delta\Delta$ Ct method.

Karyotype test

The normal chromosomal quantity, arrangement and morphology of hiPSC-NSCs were evaluated in order to uncover if any probable mutation occurred during cell cultivation or passaging.

Animals

Nine monkeys (weigh: 3-6 Kg, age: 3–6 years old, both genders) were enrolled in this experiment according to the ROYAN Institute Review Board and Ethics Committee's guidelines

(IR.ACECR.ROYAN.REC.1397.104), (approval ID: EC/92/1009). Total animals were quarantined for one month while tested negative for microbial and viral infections (intestinal parasites, tuberculosis, and Simian Immunodeficiency Virus (SIV), herpes B, hepatitis A, and B virus) prior to the experiments. Animal subjects were randomly grouped into control ($n = 2$) and hiPSC-NSCs Transplanted ($n = 7$). Ten days post-injury (PI), concurrent with sub-acute phase initiation in injured animals, hiPSC-NSCs and PBS were injected intra-spinally into Transplanted and Control subjects, respectively. Thereafter, behavioral improvements were assessed through six months post-transplantation (PT) in all animals.

Contusive SCI modeling, animal care, and cell transplantation

In this study, the SCI contusive model was achieved as described in our preview report (23). Briefly, animal anesthesia was performed by intramuscular injection of Ketamine (15 mg/kg) and Xylazine (0.4 mg/kg). The spinal cord was exposed through laminectomy at the T9-T10 segment. Simultaneously, the upper and lower spinous processes were immobilized so that the desired area (about 10 mm²) became completely available for weight drop. Eventually, a 50-gram weight was released from a 12-cm height (using a modified NYU impactor) on the spinal cord to achieve the contusive model. Bladder discharge was performed manually as long as the animal

demanded it, which is usually around 3–5 days PI (24). If the clinician suspected that the animals were in pain during bladder discharge, he would inject them Tramadol (20 mg/kg) intramuscularly. Similar to every surgery procedure (on humans or monkeys), the animals were instructed to follow the Nothing by Mouth (NPO) protocol from eight hours before SCI to eight hours PI. Therefore, animals did not suffer stool retention in the early hours of surgery, and they were given Lactulose syrup for several days (1-2 cc every 12 hours) if any difficulty in defecating had been observed. Cefazolin (25 mg/kg) was injected into the animals twice a day for a week, PI. Thereafter, about 2×10^7 hiPSC-NSCs (diluted in 50 μ l PBS) and 50 μ l PBS were injected intra-spinally to the Transplanted and Control subjects, respectively, as follows: using a Hamilton syringe (50 μ L, 27G); via an automated micro syringe pump (Stoelting, USA); under similar surgical procedure to the SCI modeling; ten days PI. Cyclosporin A (20 mg/kg, Sandimmune) was injected intramuscularly into the total animals in both groups) once a day to avoid immunological rejection throughout the whole experiment.

Magnetic resonance imaging (MRI)

AnMRI was performed in order to visualize the spinal cord anatomy, lesion site, cavity formation, bleeding, and vacuolization one week before and after SCI in addition to six months PT. For this aim, anesthetized monkeys (using 15 mg/Kg Ketamine and 0.4 mg/kg Xylazine) were positioned in a 3-Tesla superconducting scanner (Siemens, Prisma, Germany) to capture sagittal and axial (T1 and T2-weighted) MR scans (25).

Behavioral assessments

Monthly sensory and motor behavioral improvement of all animals was evaluated by two blind neurologists according to needle stimulation as well as the Tarlov's scale, during a six-month time-course(23). Animals who were unable to move their hind limb joints (ankle, knee and hip) freely would be scored as 0, whereas, individuals with improved mobility would be given more enhanced scores of 4 (Tarlov's scale). Additionally, the joint turning direction to distinguish the flexion and extension

gestures was observed and scored elaborately. The toe sensitivity to painful stimulation or touch was evaluated by pinching forceps, also, the anal, plantar and knee reflexes were identified as animal responses to a needle simulation and responses were scored between 0-4(26).

Histological assessments

At six months PT, animals in both groups were sacrificed and perfused with 4% (w/v) paraformaldehyde in phosphate-buffered saline. The spinal cord was post-fixed overnight in 4% (w/v) paraformaldehyde and cryoprotected with 30% sucrose at 4 °C for 48 h. Tissues were embedded in an optimal cutting temperature compound (OCT) and sectioned with 12 µm thickness. Sections were placed into a hematoxylin working solution to stain the nuclei,

and then Eosin was used to stain the cytoplasmic structure. Collagen fiber was stained via 1% phosphomolybdic acid and fast green solution (1-3 minutes). Imaging was performed using light microscopy (OLYMPUS, BX51, Japan). LFB staining was employed for both transplanted and control groups after six months. Frozen sections of the epicenter, 0.5 and 1 mm rostral and caudal to the lesion site, were located into 1:1 alcohol/chloroform to be de-fatted. Then, the sections were placed in 95% ethyl alcohol to become rehydrated. Sections were left in the LFB solution at 56° C for 16 hours. Next, samples were rinsed off with 95% ethyl alcohol and finally, were placed in the lithium carbonate solution for 30 seconds. Mounted slides were visualized under light microscope (OLYMPUS, BX51, Japan).

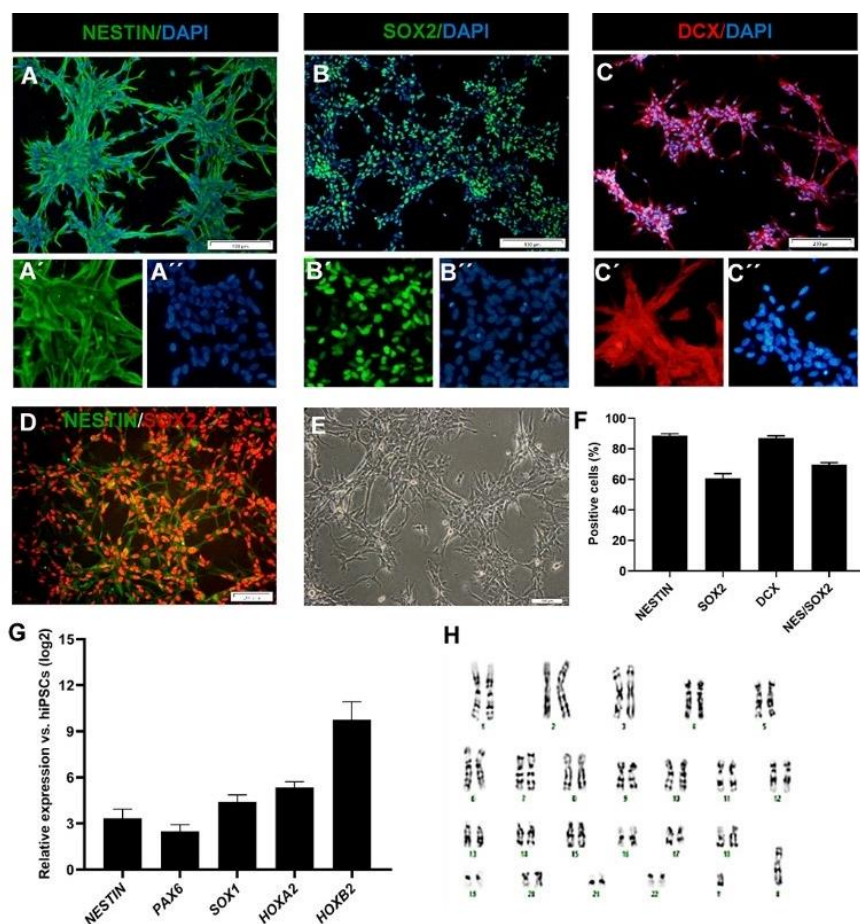


Figure1. hiPSC-NSCs characterization. hiPSC-NSCs expressed A) NESTIN, B) SOX2, and C) DCX as NSCs-specific markers and was able to D) Co-express the NESTIN and SOX2. E) Morphological properties of the hiPSC-NSCs are shown by phase contrast imaging. F) The protein expression of the NSC markers were quantified. G) Neural stem cell gene expression patterns were evaluated in hiPSC-NSCs by qPCR, demonstrate that this cell source is capable of *NESTIN*, *PAX6*, *SOX1*, *HOXA2*, *HOB2* expression. H) G-banding analysis for hiPSC-NSC. (Scale bars for A = 100 µm, B and C = 200 µm, D and E = 100 µm).

hiPSC-NSCs were efficient in improving spinal cord anatomical deficits

MRI was employed to visualize the anatomical structure of the spine, ependymal canal and surrounding tissue in every individual before injury, PI and PT. Every individual in this study was diagnosed with a normal spinal cord and intact ependymal canal before SCI. Hemorrhage, edema and spinal cord contusions are clearly detectable at the lesion site in subsequent SCI and one-week PI. On the other hand, the abnormal condition of the spinal cord was ameliorated through hiPSC-NSCs treatment in Transplanted monkeys in comparison to the Control, six-month PT (Fig. 2).

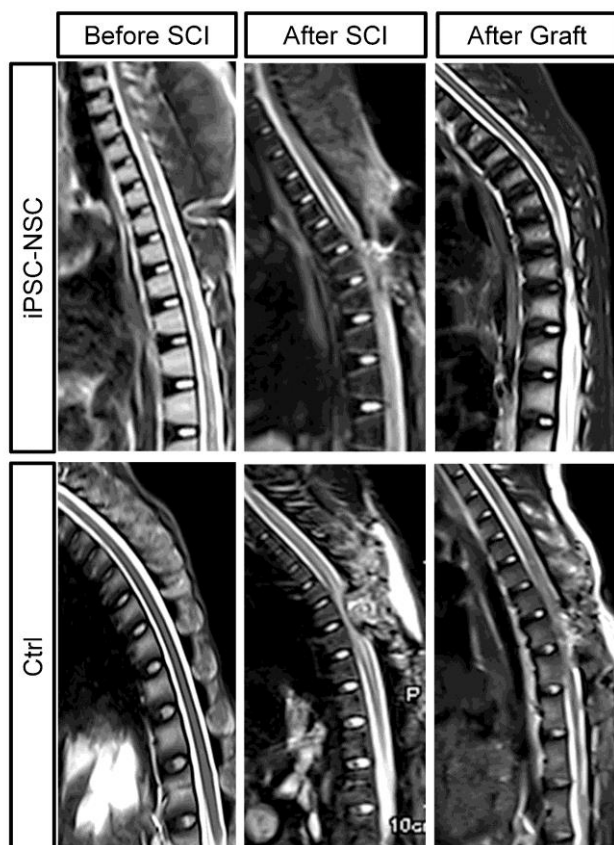


Figure 2. Magnetic resonance imaging (MRI). Spinal cord anatomy, ependymal canal and dura are depicted before (left column), post-injury (middle column) and six months after hiPSC-NSCs transplantation (right column) in experimental and Control group at T9-T11 level.

hiPSC-NSCs were sufficient for sensory and motor recovery

The sensory ability of the monkeys was evaluated by means of animal reactions to toe

pinches as well as ankle, knee and hip joints responses to their own body position, i.e. proprioceptive inputs. According to our observations and dedicated scores, sensory responses were clearly elevated during the second and third months of PT in the transplanted group, whereas, these responses appeared in Control mostly after the fifth month, with no significant differences (one-way ANOVA, post-test Tukey, NS, Fig. 3A-D). Moreover, the anal reflex, evoked by needle stimulation, was returned in transplanted animals at the first month, however, was not improved in Control until the fifth month. There was no significant difference between these two groups (one-way ANOVA, post-test Tukey, NS, and Fig. 3E). The plantar reflex to the needle stimulation, on the other hand, was evoked in Transplanted and Control monkeys in the fourth and fifth months of PT, respectively, with no remarkable differences (one-way ANOVA, post-test Tukey, NS, and Fig. 3F).

To address the motor improvement, Tarlov's scale was adapted to quantify the clinical signs of monkeys' movement by two blind neurologists. During the first month, total animals revealed absolute immobility of hind limbs in consequent of SCI, thus, they were identified as entirely paraplegic, and their Tarlove's scale was zero. Thereafter, by the second month PT, Transplanted monkeys showed perceptible movement of one to three joints of the hind limb, and their Tarlove's scale was 1, while the primary signs of movement were observable in control only from the fifth month. Indeed, the considerable recovery of the motor ability was initiated from the fourth month PT in Transplanted monkeys by hind limb joint movements and gradually approached to weight support enhancement as well as better assisted crawling (Tarlov's scale 3-4). However, the noticeable activity of one to three hind limb joints was roughly detectable in Control after six months PT, which lead to a significant contrast of motor recovery between these two groups during the last three months of investigations (month four to six) (one-way ANOVA, post-test Tukey, $P < 0.01$, $P < 0.001$, Fig. 3G).

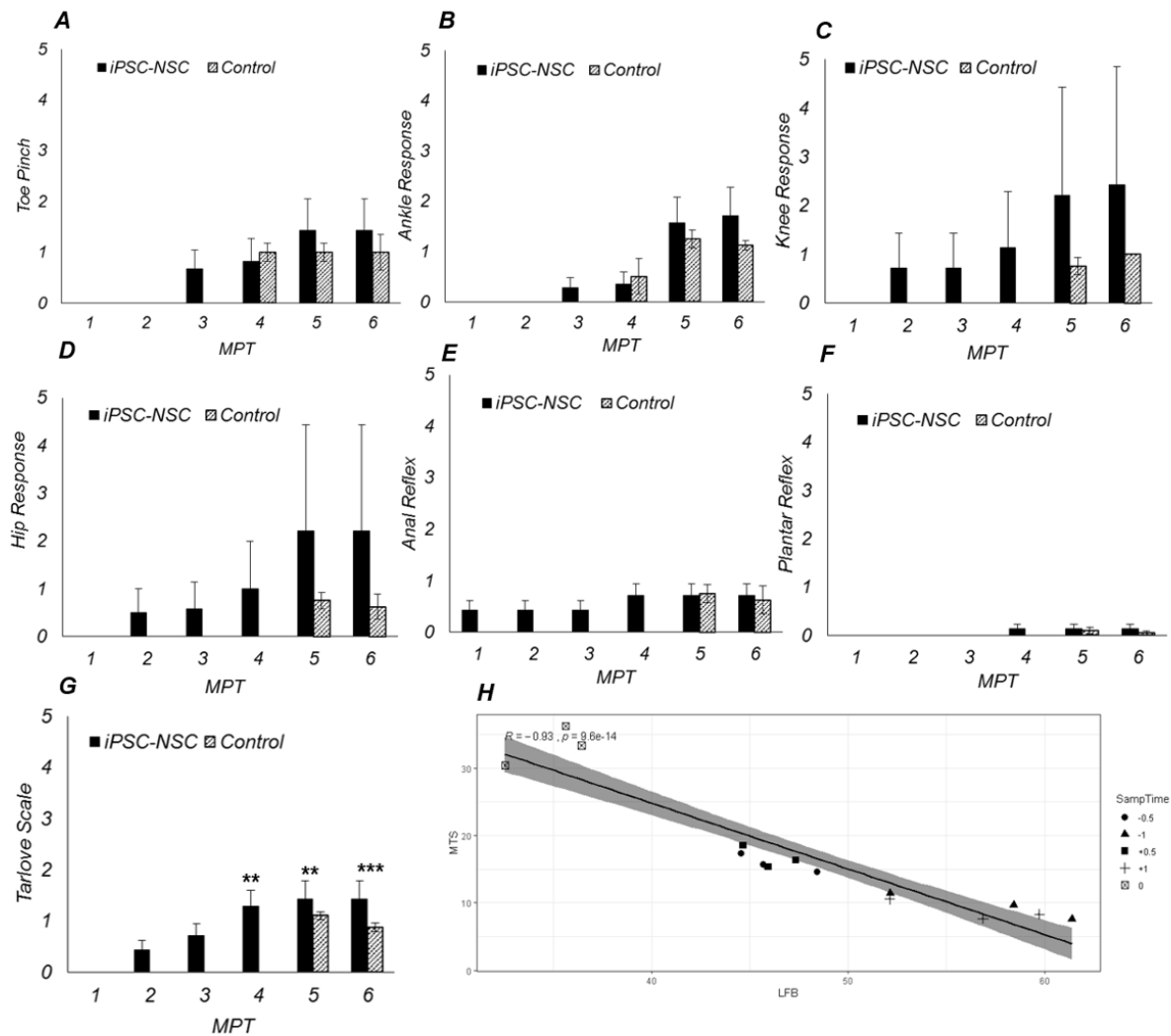
Fig. 3


Figure 3. Sensory and motor behavioral outcome. A-F) The sensory responses in toe, ankle, hip, knee and anus, evoked by pinching, were not improved significantly during six months, however, Transplanted individuals showed more robust responses in earlier months. G) Motor skills were evaluated by Tarlov's scale. Animals treated with hiPSC-NSCs showed more significant enhancement in motor performance after six months (Means $\pm$ SD, A p-value < 0.05 , one-way ANOVA with Tukey's post hoc tests) (MPT: Month Post Transplantation). H) Correlation coefficient between LFB and MTS positive area demonstrated the negative relationship between Fibrotic scar reduction and remyelination enhancement in the epicenter, 0.5, as well as 1 mm rostral and caudal distances from the lesion site.

hiPSC-NSC causes fibrotic scar reduction and promotes myelination

To address the effect of hiPSC-NSCs transplantation on fibrotic scar reduction, MTS was performed on transverse sections from the epicenter (lesion site) to 0.5 and 1 mm rostral and caudal distances of the lesion zone. According to the MTS, the percentage of collagen volume, representative of fibrotic scar, to the total mass is considerably reduced after six months of PT in injured animals throughout the

lesion zone (from epicenter to both rostral and caudal directions in comparison to control (one-way ANOVA, post-test Tukey, $P < 0.01$, $P < 0.001$, Fig. 4A and C). The hiPSC-NSCs contribution to myelin repair was contained via LFB staining in Transplanted monkeys after six months PT as well. Consistent with fibrotic scar restriction, a substantial increase in myelination was also determined in Transplanted monkeys compared to the Control (one-way ANOVA, post-test Tukey, $P < 0.01$, $P < 0.001$, Fig. 3H and 4B and D).

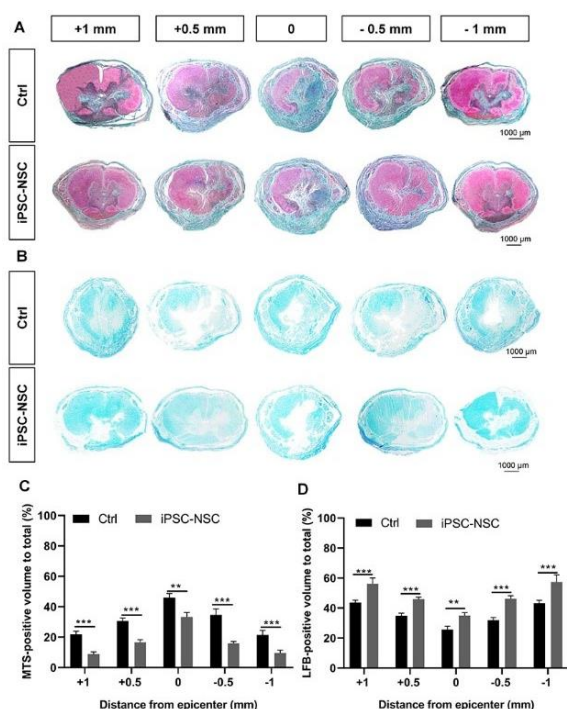


Figure 4. Histological analysis. A-B) Fibrotic scar (collagen density) reduction and myelination enhancement were assessed by MTS and LFB respectively, from epicenter (0) to 0.5 mm, and 1 mm rostral and caudal of the lesion site. C-D) The quantification results of MTS and LFB reveal the ameliorative effect of hiPSC-NSCs in fibrotic scar limitation and myelination increase (Means $\pm$ SD, A p-value < 0.05, one-way ANOVA with Tukey's post hoc tests).

DISCUSSION

In the present study, we mainly aim to address the behavioral outcome of hiPSC-NSCs intra-spinal transplantation by evaluating sensory perception and motor activity throughout a six-month timeline, in seven spinal cord injured Rhesus monkeys. The asymmetrical distribution of subjects in the Transplanted (n=7) and Control (n=2) groups, as two statistical frames, was chosen to better interpret the impact of hiPSC-NSCs on sensory/motor improvement. Herein, the cell source possesses NSCs' dedicated gene (*NESTIN*, *SOX1*, and *PAX6*) and protein (*NESTIN*, *SOX2* and *DCX*) profiles. The nominated hiPSC-NSCs also identified to have *HOXA2* and *HOXB2* expression, suggesting they are more reliable for spinal cord treatment. Considering our results, improved sensory responses as well as reflexes triggered by pinching or using a needle, were remarkably

raised early from the first or second month of hiPSC-NSCs treatment, while this ability was absent in non-treated subjects for a longer time until the fifth month. Additionally, significant motor activity, comprising improved weight support and joint movement, was achieved in the fourth month of study in Transplanted individuals. The mentioned behavioral recovery was also consistent with spinal cord anatomical improvement, as depicted by MRI during six months of treatment.

Spinal cord injury may cause permanent deficiencies in organs below the level of injury, while the patients also suffer from a more temporary situation so called as "spinal shock". This immediate circumstance is distinguished by severe paralysis, loss of muscle tone, and reversible depression of all or most of the spinal reflexes in addition to hindlimb sensation (27). The duration and severity of spinal shock differ between species; in monkeys, it lasts from two to several more weeks. Whenever the spinal cord passes the shock, several spinal and cutaneous reflexes ought to be returned (28,29). Our results highlighted that hiPSC-NSCs efficiently diminished the duration of spinal shock by recovering most of the sensory reflexes. Additionally, according to Tarlov's scale, transplanted animals exhibited hind-limb movements against gravity but were unable to support weight in the second month. Indeed, their mobility progressed gradually to reach the standing capability and crawling (with assistance) in the last five to six months, whereas control subjects could barely stand up half way. In our previous experiment, transplantation of adult NSCs, derived from sub-ventricular zone of the brain, into similar contusive SCI primate models led to analogous motor ability improvement in seven months (26). Besides, NSCs, derived from other sources, were able to positively modify the motor impairment in SCI (in cervical level) marmoset monkeys (30). Therefore, our findings regarding hiPSC-NSCs substantial ameliorative influence of hiPSC-NSCs on motor and sensory behavior in injured rhesus monkeys are in line with other studies in which NSCs from various sources were employed.

Spinal contusion will disconnect the reciprocal communication between the central nervous system and limbs, which will destroy muscle innervation and give rise to muscle atrophy. A fibrotic scar gradually develops at the injury site due to the perivascular fibroblast's unfavorable production and collagen accumulation inside the created cavity (31), which may exacerbate the atrophy. Accordingly, restricting the volume of spinal cord fibrosis, in animal models and humans, would provide better sensory-motor communication and inhibit progressive muscle atrophy (28). Here, the fibrotic scar size become considerably restricted over six months, and muscle atrophy was rarely found in transplanted monkeys, implying the effectiveness of hiPSC-NSCs. Also, the myelination enhancement in transplanted animals, in association with scar reduction, suggests that the neural circuit is modified at the injury location as well. In fact, the negative correlation between MTS- and LFB-Positive areas (less than zero) in the transplanted group, indicated a strong relationship between fibrotic scar shrinkage and remyelination at the injured site and its rostral and caudal vicinities. In other words, it seems that the myelination increased as the fibrotic scar decreased six months PT. Fibrotic scars generally discourage myelination, even in other neurogenerative diseases such as multiple sclerosis (32). Simultaneous progressive demyelination, induced by spinal trauma, may irreversibly change neuroplasticity in the entire CNS as well (33). Therefore, anti-fibrotic mechanisms may serve as promising therapeutic targets to rescue myelination with an increased consequent signal propagation in newborn neurons following the trauma (34,35). According to our findings, hiPSC-NSCs were adequate to either reinforce anti-fibrotic procedures, myelination as well as atrophy elimination. Although the monkeys in this study were not physically rehabilitated to specifically determine the exclusive role of hiPSC-NSCs in animal function, the combinatorial approach is recommended to achieve a more appreciable recovery (36).

Overall, we believe that the SCI imposes sensory/motor debilitations that may render a person dependent on caregivers, therefore,

assistive approach requires facilitating the perception of touch, mobility and self-care in patients. hiPSC-NSCs, as an autologous cell source (especially for humans), have already been found to be appropriate to rectify neuronal and oligodendrocyte loss (10) and here we propose that they are adequate for sensory and motor improvement in non-human primate as well.

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DISCLOSURE

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Razieh Jaberri performed *in vitro* cultivation of hiPSC-NSCs, cell and histological staining, figure preparation, data collection, statistical analysis and contributed in manuscript writing. Reza Jabbari performed SCI modeling and hiPSC-NSCs transplantation in addition to behavioral and clinical assessments, Shiva Nemati performed RT-PCR and provided hiPSC-NSCs for transplantation. Sara Mirsadeghi wrote the paper and was the main English editor. Soroush Mohimafi and Mostafa Hajinasrollah was the veterinarian surgeons and performed animal caring as well as clinical assessments. Behrouz Rafiei and Hossein Ghanaati, performed MRI and further MRI evaluations. Sahar Kiani was the main innovator of the proposed hypothesis, supervised the whole project, discovered the grant sources and contributed in manuscript writing-editing, statistical analysis and prepared behavioral figure.

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