Combinational therapy of lithium and human neural stem cells in rat spinal cord contusion model

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Abstract
A large number of treatment approaches have been used for spinal cord injury improvement, a medically incurable disorder, and subsequently stem cell transplantation appears to be a promising strategy. The main objective of this study is to ascertain whether combinational therapy of human neural stem cells (hNSCs) together with lithium chloride improves cell survival, proliferation, and differentiation in a rat spinal contusion model, or not.

Contusive spinal cord injury was implemented on Wistar male rats. Experimental groups comprised of: control, hNSCs transplanted, lithium chloride (Li), and hNSCs and lithium chloride (hNSCs + Li). In every experimental group, locomotor activity score and motor evoked potential (MEP) were performed to evaluate motor recovery as well as histological assessments to determine mechanisms of improvement.

In accordance with our results, the hNSCs + Li and the Li groups showed significant improvement in locomotor scores and MEP. Also, Histological assessments revealed that transplanted hNSCs are capable of differentiation and migration along the spinal cord. Although NESTIN-positive cells were proliferated significantly in the Lithium group in comparison with control and the hNSCs + Li groups, the quantity of ED1 cells in the hNSCs + Li was significantly larger than the other two groups.

Our results demonstrate that combinational therapy of hNSCs with lithium chloride and lithium chloride individually are adequate for ameliorating more than partial functional recovery and endogenous repair in spinal cord-injured rats.

Keywords: contusion, derived neural stem cells, hNSC, lithium chloride, spinal cord injury

1 | INTRODUCTION

Spinal cord injury (SCI) is a traumatic situation leading to persistent neurological dysfunctions (Siddiqui, Khazaei, & Fehlings, 2015; Su, Niu, Liu, Zou, & Zhang, 2014). The prevalence of SCI has reported almost 2.1–130.7 incidents per million per year, which is a considerable figure in developing countries in 2013 that mostly contains young men (Rahimi-Movaghar et al., 2013). Many research and clinical approaches based on rehabilitation, pharmacological treatments, tissue engineering, and neural cell replacement were suggested for SCI repair but none of them, solely, could pave the way for a reliable cure, however, they introduced a starting point to discover a promising cure (Côté, Murray,
Injured spinal cord, contains traumatic agents that will replace to glial scar formation and subsequent neuronal degeneration along with devastating inflammation. Indeed, inflammation would be propagated throughout the spinal cord tissue causing issues even for transplanted cells to survive and regenerate tissue losses (Cregg et al., 2014). In this sense, amongst all combinational therapy approaches, specifically, cell transplantation in association with a protective chemical are becoming more acceptable and promising for repairing injured tissue (Lin et al., 2016). Although, some of the chemicals may be responsible for beneficial effects on cells and tissue microenvironment, however, others are most effective for neuronal protection and suppressing the inflammation. Besides, nominating a qualified cell source as an alternative for lost neural cells is a substantial job to do. Thus, this is important to combine appropriate cell type and chemical with each other to yield reasonable improvement in SCI repair (Assinc, Duncan, Hilton, Plemel, & Tetzlaff, 2017; Muheremu, Peng, & Ao, 2016).

Neural stem cells (NSCs) are multipotent and self-renewal cells, capable of both neuronal and glial generation (Filippis & Binda, 2012). NSCs are residents of unique and particular regions in the mammalian central nervous system namely: subventricular zone (SVZ) and dentate gyrus (Hirie & Alvarez-Buylla, 2011; Rolando et al., 2016). In addition to their original sources, NSCs also can be produced and differentiated in vitro from pluripotent embryonic stem cells (ES; Jing et al., 2014; Tsang et al., 2013). Recent studies indicated that transplantation of NSCs to spinal cord-injured animal models may rewire the damaged tissue. They also introduced cell transplantation as a novel strategy for manipulation of damaged environment due to NSCs secreting neurotrophic factors such as BDNF, and GDNF (Salewski, Mitchell, Shen, & Fehlings, 2014; Zhang, Tu, Zhang, & Shen, 2014).

Transplanting neural stem cells construct significant improvement in spinal cord-injured animal models, especially, at the acute phase of injury by means of axonal growth promotion as well as lost cell replacement (Cheng et al., 2017; Iwanami et al., 2005; Lu, Jones, Snyder, & Tusznynski, 2003). Thus, the low efficiency of NSCs transplantation is a challenging problem (Lin et al., 2016), whereas, combinational therapy, the chemical application along with NSCs, may provide a protective circumstance for transplanted NSCs survival and operation (Hosseini et al., 2018; Lane, Lepore, & Fischer, 2017; Song, Peng, & Ye, 2015; Zhang & Shen, 2015).

Studies demonstrated that lithium is able to interfere in several cell signaling pathways associated with neurotrophic factor secretion (Chiu & Chuang, 2010). Lithium inhibits inositol monophosphatase enzyme, in turn, prevents the cell apoptosis (Oruch, Elderbi, Khattab, Pryme, & Lund, 2014). Also, lithium plays a role in promoting neuronal plasticity by influencing on N-methyl-D-aspartate receptors (NMDA-R) as well as nitric oxide pathway (Chiu & Chuang, 2010; Oruch et al., 2014). In addition, lithium is capable of inhibiting glycogen synthase kinase 3 beta which decreases the apoptotic factors (Chiu & Chuang, 2010; Hashimoto, Hough, Nakazawa, Yamamoto, & Chuang, 2002). Other studies also claimed lithium enhances the proliferation, survival, and neuronal differentiation of neural progenitor cells both in vitro and in vivo (Su, Chu, & Wu, 2007; Young, 2009).

Studies showed combining treatments can develop the improvement in reducing the difficulties caused by SCI (Olson, 2013). Therefore, combining lithium properties with NSCs appears to be an appropriate option to enhance the efficiency of neural stem cell transplantation into an injured spinal cord. Oral administration of lithium carbonate is not capable of substantial changes in functional outcomes in patients with SCI who suffer chronic phase (Esfandiari et al., 2012; Wong et al., 2011).

Our results demonstrate that combinational therapy of hNSCs with Li chloride and Li chloride individually are adequate for ameliorating more than partial functional recovery and endogenous repair in spinal cord-injured rats.

2 MATERIAL AND METHODS

All procedures involved animal subjects have been approved by the institutional animal care and use committee (IACUC) at ROYAN institute, Iran. A total number of 123 adult Wistar Rat were involved in this study.

2.1 Animals

Adult male Wistar rats (250–280 g) were maintained individually or doubled under standard housing conditions (22 ± 2°C, available food and water, 12 hr light/dark cycle). After spinal cord injury surgery dextrose saline (5 ml) for 5 days and enrofloxacin (5 mg/kg) for 10 days were injected. The bladder massage was continued until they regained full bladder control. Cyclosporine A solution (10 mg/kg) was administered subcutaneously every day from 5 days after SCI in all groups to prevent immunosuppression (Figure 1a).

2.2 Electrode implantation and electrophysiological assessment

One week before SCI surgery, rats were anesthetized with the cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). Heads were fixed in a stereotaxic device (Stoelting, Wood Dale, IL). An incision was made along the midline of the skull and two holes were drilled with the standard dental drill (Faro, Lake Mary, FL) in the left hemisphere. The first hole was located 2 mm anterior to the Bregma and 2 mm lateral to the midline (motor cortex and evoked electrode). The second hole was located 6 mm posterior to the Bregma and 4 mm lateral to the midline (reference electrode). Screw electrodes were implanted in a depth of 0.75 mm to have contact with cortex, without any forced pressure to dura mater. Carbosylate dental cement (Acropars, Iran) was used to fix electrodes.

Recording and reference needle electrodes were inserted intramuscularly into the middle of the tibial anterior muscle of the right hindlimb and the ankle part of the same muscle respectively. A footpad was attached as the ground electrode. Stimulation and record were
performed with an Electro-module device (R12, ScienceBeam, Tehran, Iran). The motor cortex was stimulated with 15 trial comprising five pulses. The pulse duration was 200 µs with 1 mA intensity and 15 Hz frequency. MEP recording was performed two times at the first and fourth weeks after SCI.

### 2.3 Spinal cord injury surgery

After anesthetization with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), posterior part of animal waste was shaved and incise along the body midline. Laminectomy was performed on T10 level with a dental drill (Faro) without any inflict to the dura. T9 and T11 spinous processes were fixed with clipless. The 10 g rod was released from 25 mm height on the exposed spinal cord via NYU-impactor. After the injury, connective tissue and skin were sutured. Animals were placed on 37°C plates for recovery.

### 2.4 Expansion and characterization of hNSCs

GFP-positive hNSCs were differentiated from Royan H6 line (human ESC-derived NSCs; Baharvand et al., 2006; Nemati et al., 2010). The cells initiate a continuous proliferation in the presence of bFGF.
(40 ng/ml; Royan Biotech, Tehran, Iran), EGF (20 ng/ml), knockout serum replacement (5%, Gibco, MA), N2 supplement (1%, Gibco), and B27 supplement on poly-L-ornithine/laminin-coated dishes. NSCs then were transferred to other coated plates after 90% confluency. After characterization of hNSCs, they were infected by viral vectors carrying Lenti-CMV-GFP and GFP-positive cells further harvested for transplantation.

2.5 | hNSCs transplantation and lithium treatment

All treatments began 1 week after SCI at subacute phase (Figure 1a). We represented four experimental groups in this study. Animals in the control group experienced only needle stress on their spinal cord under the anesthetic surgery. hNSCs were transplanted into rat spinal cord in the second group (hNSCs group). Li chloride (85 mg/kg body weight, dissolved in distilled water, Sigma-Aldrich, St. Louis, MO) were injected intraperitoneally once a day for 14 days in the third group (Li group). Animals of the fourth group received a combination of both hNSCs and Li (hNSCs + Li group).

For hNSCs transplantation, the animal spinal cord was exposed again so that one million hNSCs diluted in Dulbecco’s modified Eagle medium (DMEM)/F12 were injected by Hamilton syringe (27G) in the needle before withdrawal to minimize cell leakage.

2.6 | Open-field activity

Rats were placed in a plexi box (100 [length] × 100 [width] cm × 20 [height]; Luedtke et al., 2014) weekly for general locomotion assessments using the Basso, Beattie, Breshman (BBB) Scale (Basso, Beattie, & Bresnahan, 1996).

2.7 | Immunocytofluorescence staining

Cultured cells were fixed with 4% paraformaldehyde for 15–20 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 30 min at room temperature. Afterward, the cells were stained with primary antibodies in blocking solution at 4°C overnight. After that, samples were incubated with secondary antibodies for 1 hour. Cell nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The needle was left in place for 5 min after injection before withdrawal to minimize cell leakage.

2.8 | Histological assessments

For histological assessments, some animals (four in each group) selected randomly and killed in different stages of therapy. They were perfused with 4% paraformaldehyde. One centimeter length of every spinal cord containing the lesion site in the middle part was cut out. Tissues were kept in 4% paraformaldehyde and 30% sucrose PBS solution for cryosectioning tissue-blocks and were kept in 4% paraformaldehyde for paraffin tissue blocks.

The spinal cord tissues were sectioned with 6 µm thickness according to standard cryosection protocols and sections were selected randomly to perform immunohistofluorescence staining. For paraffin tissue samples, the tissues were dehydrated, blocked, and sectioned into 6 and 10 µm thickness according to standard protocols and sections were selected randomly to perform Immunohistofluorescence staining.

2.9 | Immunohistofluorescence staining

Frozen sections, after permeabilizing and blocking in host serum, were incubated with primary antibody overnight at 4°C, and incubated with secondary antibodies for 1 hour at 37°C. Then they were incubated with DAPI for 1 minute at room temperature.

Paraffin-embedded sections were rehydrated by xylene and decreased percentages of ethanol and distilled water. Then, antigen retrieval was performed in Dako buffer in 37°C. After permeabilizing and blocking, sections were incubated with primary antibody overnight at 4°C, washed, and incubated with secondary antibodies for 1 hour at 37°C. After that, they were washed and were incubated with DAPI solution for 1 minute at room temperature and were washed up again.

The sections were analyzed with a fluorescent microscope (IX71, Olympus, Japan). For the quantification of the NESTIN and ED1-positive cells, 7–14 sagittal tissue sections were used in each group. These cells divided into DAPI-positive cells. Primary antibodies were:

NCAM (1:200, Santa Cruz, SC-8304), SOX2 (1:200, Santa Cruz, Sc-20088), NESTIN (1:200, Sigma, SAB4200347)

STEM121 (1:200, Clontec, Kyoto, Japan, Y40410), GLAST (1:200, Abcam, Cambridge, UK, Ab41751), NG2 (1:200, Millpor, Burlington, MA, AB5320), β Tubulin (1:200, Santa Cruz, Sc-86255), NESTIN (1:200, Chemicon, MAB353), GFAP (1:200, Sigma, G3893), and ED1 (1:100, ABserotec, Kidlington, UK, mca341r). Nuclei were counterstained with DAPI (0.1 µg/ml, Sigma-Aldrich, D8417).

2.10 | Data analysis

Electrophysiological data were analyzed with Scilab software (version 6.0.0; http://scilab.io/company/careers/, Rungis, France). The ImageJ software (v1.51k version; imagej.net) was used for counting the nuclei of tissue sections. Statistical analysis was performed by SPSS (version 16). One-way analysis of variance (ANOVA) and Tukey posttest were implemented to compare the recovery of the groups with the number of more than 10. Within-group data were analyzed with paired Student’s t-test and between-group data was analyzed with unpaired the Student’s t-test. All data has been presented by mean ± SEM or ± SD.

In histological quantifications, NESTIN and ED1-positive cells were counted manually by immunofluorescence microscopy in 7–14 slides in each group (10 µm thickness and selected randomly). Then the number of positive cells of each slide divided into DAPI-positive nucleus which was counted by the ImageJ software (version 1.51k). Then the proportion analyzed statistically.
3 | RESULTS

3.1 | Characterization of hNSCs

In this study, NSCs have been recognized by stemness/progenitor state markers such as Nestin, SOX2, and NCAM. The intermediate filament protein Nestin, a multipotency marker, expression was more than 90%. SOX2 as the most critical proliferation regulator and NCAM were highly expressed in NSCs as well (Basso et al., 1996; Luedtke et al., 2014; Figure 1b). Characterized NSCs were GFP labeled via the lentiviral delivery system to become detectable in spinal tissue (Figure 1c).

3.2 | SCI model confirmation

To confirm SCI model hematoxylin and eosin (H&E) staining were implemented in sagittal and transverse spinal cord sections 7 days after the SCI surgery. H&E manifested tissue damage plus neuronal degeneration and cell elimination (Figure 2a,b). Besides, expression of rat glial fibrillary acidic protein (GFAP) around the lesion site shows initiation of astrogliosis as a specific landmark of contusion model (Figure 2c).

3.3 | Behavioral assessment

To examine functional recovery after SCI, the BBB test was performed weekly (n = 12 in the control group, n = 9 in the hNSCs group, n = 10 in the Li group, and n = 10 in the hNSCs + Li group: Figure 3a). The BBB score of all animals was determined 21 before the SCI surgery. This score shifted to zero the day after the surgery and remained the same at least for 3 days.

Treated animals demonstrated a significant difference in functional recovery in comparison with control animals since the fourth week (p < 0.01 in the Li and the hNSCs + Li groups, p < 0.05 at Week 4 and 5 and p < 0.01 at Week 6 and 7 in the hNSCs group). Also, the Li and the hNSCs + Li groups showed a significant difference in BBB score at third week (p < 0.05). The Li group and the hNSCs + Li group acquired different BBB score significantly in sixth and seventh week respectively (p < 0.05 in Week 6 and p < 0.01 in Week 7).

3.4 | Motor evoked potential recording

Motor evoked potential (MEP) recording was performed three times (1 week before and after SCI and in the fourth week) in this study to ascertain the value of recovery in spinal tracts (n = 2 in the control group, n = 4 in the hNSCs group, n = 5 in the Li group, and n = 6 in the hNSCs + Li group). In recording procedure, left motor cortex was stimulated and recording was performed from right tibia muscle. In baseline traces, we are able to observe two separated waves in consequence (Figure 3b). The first wave is called N1 and the second one is N2 (Redondo-Castro, Navarro, & García-Alias1, 2016). Usually, both waves were disappeared 1 week after SCI or sometimes their amplitude was decreased, however, most of the waves would return 4 weeks after SCI.

Amplitude, latency and signal duration of N1 and N2 waves were analyzed and compared between experimental groups. All data are normalized to the baseline recording of each animal.

The amplitude of both waves in the Li group improved significantly in the fourth week in comparison with the first week (p < 0.01 in N1 and p < 0.05 in N2). The N1 and N2 latency in Li and the hNSCs + Li group at Week 4 improved significantly in comparison with Week 1 (p < 0.05 in N1 in both groups, p < 0.01 in N2 of the Li group, and p < 0.05 in N2 of the hNSCs + Li group).

The N2 duration in the Li and the hNSCs + Li groups at first week manifest a significant difference toward Week 4 (p < 0.05). Also, there are significant differences in N1 latency between the control and the Li groups in Week 4 (p < 0.05). N2 latency between the hNSCs and the Li groups in Week 4 (p < 0.05), N1 Duration between the control and the hNSCs groups in Week 1 (p < 0.05), N1 Duration between hNSCs and the Li groups in Week 1 (p < 0.05), and N2 Duration between the control and the Li groups in Week 4 (p < 0.05; Figure 3c).

3.5 | The fate of transplanted hNSCs in injured spinal cord

To investigate hNSCs survival in spinal cord tissue, two animals from each hNSCs and the hNSCs + Li group were killed at fourth and seventh weeks for spinal tissue extraction. The cryosections were immunostained with an anti-GFP antibody, enable us to detect the hNSCs presence in every tissue. In fact, labeled hNSCs were observed only in Week 4 rather than in Week 7. Because of that, we had to labeled STEM121 marker for hNSCs to distinguish human sourced cells from rat origin. Surprisingly, we found human cells approximately 4mm farther than injection site reflecting NSCs migration in a rostrocaudal direction (Figure 4). About 5–19 STEM121-positive cells were found in each random 10μm sections in both hNSCs and hNSCs groups.

The immunofluorescent staining was performed on paraffin sections of the seventh week old spinal cord tissues with human astrocyte-specific glutamate transporter (GLAST), neural/glial antigen 2 (NG2), and β tubulin antibodies that double stained with STEM121. None hNSC was observable in the control and the Li groups. However, several cells could express GLAST, NG2, and β tubulin proteins in the hNSCs and the hNSCs + Li groups, whereas they are STEM121 positive. All of these cells have observed in both rostral and caudal of the injected site with at least 1mm distance to the lesion area (Figure 5).

3.6 | Endogenous repair in injured spinal cord

There are neural stem cells resident in the subventricular zone and central canal in the central nervous system that have the ability of migration for the replacement of lost cells (Pekny & Pekna, 2014). To find these endogenous neural stem cells, immunofluorescent staining with rat NESTIN antibody was performed on spinal cord tissues 3 weeks after SCI. The percentage of NESTIN-positive cells in the Li
FIGURE 2  SCI model was confirmed by H&E, and immunohistofluorescent staining 1 week after SCI. (a) H&E stained transverse sections of the spinal cord are illustrated schematically. (a2) shows a transverse section of approximate of the 11th thoracic level of vertebrae which is intact. (a3) A transverse section of about 10th thoracic vertebrae which is in lesion site. (a4) A transverse section of about 9th thoracic vertebrae which is intact. (b) H&E stained of a sagittal section of injured spinal cord. The lesion site clearly is demonstrating a severe injury. (c) The astrocytes have surrounded the lesion site. As the illustration reveals, the number of GFAP-positive cells around the lesion is larger than farther astrocytes. H&E, hematoxylin and eosin; SCI, Spinal cord injury [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  Motor function improvement was examined by BBB score and MEP test. (a) One-way ANOVA with Tukey posttest was performed and data has been presented by means ± SEM. These signs show significant differences between groups: * in control and cell, # control and Li, € control and cell + Li, and ¥ in cell and cell + Li. Above the chart, the timeline of the experiment can be seen. At Week 0 SCI surgery and at the Week 1 hNSC transplantation in the hNSCs and the hNSCs + Li groups performed. From Week 1 to Week 3 lithium administrated to the Li and the hNSCs + Li groups. And cyclosporin injected into all animals from 2 days before transplantation to the end of the study. (b1) A baseline MEP recording waves. (b2) MEP recording 1 week after SCI. (b3) MEP recording waves 4 weeks after SCI. (c1, 2) N1 and N2 amplitudes changes (p < 0.05). (c3, 4) N1 and N2 latency changes (p < 0.05). (c5, 6) N1 and N2 duration changes (p < 0.05 and p < 0.001, paired the Student's t-test and one-way ANOVA statistical analysis, and Tukey posttest). MEP data has been presented by means ± SEM. ANOVA, analysis of variance; BBB, Basso, and Beattie, Breshman; hNSCs, human neural stem cells; MEP, motor evoked potential.
group was significantly higher than the control and the hNSCs + Li groups. Also, there is a significant difference between the percentage of NESTIN-positive cells in the control and the hNSCs + Li groups ($p < 0.001$; Figure 6a).

We aimed to explore local inflammation after hNSCs transplantation by counting the recruited macrophages (May et al., 2017). To determine the macrophage quantity, ED1 staining, as a macrophage-specific marker, was performed. The percentage of positive cells for ED1 in the cell + Li group was significantly higher than the control and the Li groups ($p < 0.05$; Figure 6b).

4 | DISCUSSION

Regarding to recent studies, NSCs transplantation in spinal cord injury is established to encompass an extensive set of aims. To name a few: replacement of missing cells such as neurons and oligodendrocytes, maintaining the remained neurons as well as apoptosis prevention. Besides, transplanted NSCs are supposed to provide an appropriate amount of neurotrophic factors to regenerate axons, advance synapse formation together with encouraging remyelination (Muheremu et al., 2016).

Herein, human neural stem cells were transplanted to spinal cord injury contusion model at subacute phase. Our data demonstrated how locomotor activities improved from the fourth week after the SCI in comparison with the control group. There were also considerable variations between duration of N1 wave in the hNSCs and the other groups which we propose it happened due to the limited number of animal subjects.

We were able to detect transplanted labeled hNSCs after 4 and 7 weeks since transplantation in spinal cord sections.

From what we found, it appears that hNSCs were capable of differentiating to GLAST, NG2, and $\beta$-tubulin expressing cells, representing their ability to differentiate into all three neural lineages. We did not aim to examine the expression level of synaptophysin or other neurotransmitters in this study and only focused on NSCs differentiation ability and their influence on animal functional recovery. In 1999, McDonald and his colleagues claimed that BBB score will increase after ESC-derived neural stem cell transplantation (McDonald et al., 1999). Other studies also presented that transplanted neural stem cells may migrate and differentiate into astrocytes, oligodendrocytes, and neurons in the injured spinal cord of mice, rat and primates, quite similar to our results (Iwanami et al., 2005; McDonald et al., 1999; Muheremu et al., 2016).
FIGURE 5  Transplanted cells were differentiated into three cell types which derive from neural stem cells. All the cells were found in 1–4 mm distance of rostral or caudal from the lesion. (a) Cells were found in the cell and the cell + Li groups and they express GLAST protein. (b) Also, some cells created into NG2-positive cells in these two groups. (c) β-Tubulin marker expressed by these two groups too [Color figure can be viewed at wileyonlinelibrary.com]
Endogenous neural stem cells and macrophages indicate endogenous repair and transplantation inflammation. (a) Nestin-positive cells were counted in proportion to the total cells of each tissue section, in three groups. (a4) The number of Nestin-positive cells was significantly higher than the other groups and also cell + Li group had more Nestin-positive cells than the control group significantly ($p < 0.001$, one-way ANOVA statistical analysis and Tukey posttest). (b) ED1-positive cells also were counted in proportion to the total cell number. (b4) The number of ED1-positive cells in cell + Li was larger than the other two groups significantly ($p < 0.001$, one-way ANOVA statistical analysis and Tukey posttest). ANOVA, analysis of variance [Color figure can be viewed at wileyonlinelibrary.com]
At the same time, there is a report associated with neurotrophic effects of lithium chloride on neural cells. For instance, lithium chloride influence on cell proliferation, differentiation, growth, and cell death suppression (Chiu & Chuang, 2010).

In this study, we observed that intraperitoneal injection of lithium chloride for 2 weeks (began a week after SCI) leads to locomotor improvement 3 weeks after SCI comparing with the control group. Elicited from MEP results, N1 wave has appeared more dominantly in comparison with N2 meaning that descending spinal tracts, in particular, subcortical tracts have been repaired during 4 weeks after NSCs transplantation and lithium intervention. However, when applying lithium chloride solely to the SCI model, N1 amplitude was increased significantly suggested that the motor unit is improved. We also observed substantial shortening in N1 latency comparing with the control animals, which confirm the rewiring of descending spinal cord tracts.

After removing lithium chloride injection, we counted rat Nestin expressing cells to clarify if lithium is capable of improving endogenous NSC proliferation. As we expected, the percentage of Nestin-positive cells were significantly increased in contrast with the control experiment, implying that lithium chloride application enables endogenous NSCs to proliferate, migrate, and replace the lost cells. It shows that there is a chance that lithium chloride increases the proliferation of exogenous NSCs. Actually, the astrocytes also express Nestin, thus, some of these cells could be astrocytes, though, the quantity of these cells were compared with the control group (Pekny & Pekna, 2014). Su and colleagues, similarly, determined lithium chloride ability of survival improvement, proliferation, and neural differentiation of spinal cord-derived neural progenitor cells both in vitro and after transplantation to the healthy spinal cord (Su et al., 2007). Also, a broad number of cell signaling pathways interfacing with this effect of lithium chloride have been proposed previously. For example, inhibition of inositol monophosphatase enzyme, inhibition of NMDA receptors, increasing cAMP, activation of several nuclear factors, and inhibition of glycogen synthetase kinase 3 beta enzyme (Hashimoto et al., 2002; Lane et al., 2017).

One of the main challenges of stem cell therapy is the survival of transplanted cells in the lesion site. In this study, we attempt to increase the efficiency of cell therapy via lithium chloride. Lithium offers a remarkable effect on endogenous repair which was investigated in the Li group. There are also studies regarding lithium striking influences on NSCs survival, proliferation, and differentiation in vitro (Su et al., 2007).

We evaluated the BBB score of animals weekly to reveal the capability of every particular treatment in behavior improvement. The hNSCs group in comparison with the control group showed a significant BBB score increment in every week. We did not observe any significant differences between the hNSCs and the lithium groups at Week 6 and 7. Irrespective to BBB scores, N1 and N2 latencies and N2 duration were increased significantly. Surprisingly, we found the largest quantity of NESTIN-positive cells in the Li treated group at Week 3 which totally negated our hypothesis, that we are going to observe the best endogenous and exogenous cell repair in Li + hNCSs. Eventually, it occurred to us that perhaps inflammation would be enhanced after the hNSCs transplantation. The immunity response of transplantation may cause extra inflammation and subsequently recruit macrophages to the injury site (Barker & Widner, 2004). To address this issue, we immunostained ED1 antigen, which can be expressed by most of the macrophage types. Thereupon, we observed that there is a significant increase in ED1-positive cells, implying to macrophage population, in the hNSCs + Li group in comparison with the control and the Li group.

These results demonstrated that the injured environment may face a drastic inflammation even after cell transplantation and cyclosporine injection will not suppress that. However, the most extensive immunosuppressive in studies is cyclosporine A (Pekny & Pekna, 2014). Consequently, blood monocytes and microglia will be recruited to the lesion site and produce functional macrophages (Barker & Widner, 2004; Pekny & Pekna, 2014).

The results of this study summarized that lithium cause’s improvement in spinal cord injury treatment individually or in combination with neural stem cell transplantation. We believe that although the lithium group had more convincing results, animals in the combinational therapy group would show more improvement if we find a way to control the inflammation.

CONFLICT OF INTERESTS
Authors declare that they have no conflict of interests.

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AUTHOR CONTRIBUTIONS
AM performed spinal cord injury surgery, hNSCs transplantation, animal caring, electrophysiological recording, figures preparation, histological assessments, behavioral experiment, and statistical analysis. Participated in experiment design, and manuscript writing. HS participated in spinal cord injury surgery, hNSCs transplantation, animal caring, behavioral experiment, and electrophysiological recording. RJ performed hNSCs culture and characterization. Participated in manuscript writing. SM participated in manuscript writing. MZ performed viral infection and participated in manuscript writing. ZN participated in electrophysiological data analysis. MGh participated in electrophysiological data analysis. HB participated in experiment design and manuscript writing. SK participated in experiment design, manuscript writing, and research supervision. All authors performed editing and approving the final version of this article submission.

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