Original Article

Stem Cell INVESTIGATION

Optimization of time for neural stem cells transplantation for brain stroke in rats

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Background: Despite encouraging data in terms of neurological outcome, stem cell based therapy for ischemic stroke in experimental models and human patients is still hampered by multiple as yet un-optimized variables, i.e., time of intervention, that significantly influence the prognosis. The aim of the present study was to delineate the optimum time for neural stem cells (NSCs) transplantation after ischemic stroke.

Methods: The NSCs were isolated from 14 days embryo rat ganglion eminence and were cultured in NSA medium (neurobasal medium, 2% B27, 1% N2, bFGF 10 ng/mL, EGF 20 ng/mL and 1% pen/strep). The cells were characterized for tri-lineage differentiation by immunocytochemistry for tubulin-III, Olig2 and GFAP expression for neurons, oligodendrocytes and astrocyte respectively. The NSCs at passage 3 were injected intraventricularly in a rodent model of middle-cerebral artery occlusion (MCAO) on stipulated time points of 1 & 12 h, and 1, 3, 5 and 7 days after ischemic stroke. The animals were euthanized on day 28 after their respective treatment.

Results: dUTP nick end labeling (TUNEL) assay and Caspase assay showed significantly reduced number of apoptotic cells on day 3 treated animals as compared to the other treatment groups of animals. The neurological outcome showed that the group which received NSCs 3 days after brain ischemia had the best neurological performance.

Conclusions: The optimum time for NSCs transplantation was day 3 after ischemic stroke in terms of attenuation of ischemic zone expansion and better preserved neurological performance.

Keywords: Experimental; neural; stem cells; stroke; transplantation

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Introduction

According to 2016 statistical data from American Stroke Association, brain stroke is ranked one the major cause of death after cardiovascular pathologies and cancer, and projections show that by 2030, 3.4 million more people aged 18 and above will suffer from stroke that will amount to a staggering 20% increase as compared to 2012 (1). Despite successful pharmacological intervention using thrombolytic therapy, the procedural complications combined with very narrow therapeutic time-window for intervention make it worthwhile for no more than 7% of the patients (2). The situation therefore warrants the development of alternative methods of therapeutic intervention.

Recent advancements in stem cell therapy approach have shifted the focus for the management of stroke from neuroprotection to neurorestoration that seeks to replace the damaged cells in the affected-brain with morpho-functionally competent substitute cells. Encouraging data have been reported in the experimental animal models of ischemic stroke using a distinct selection of stem cells of

various origins. Noticeable amongst these are neural stem cells (3,4), bone marrow stem cells (3,5,6), cord blood derived MSCs (7,8), adipose tissue derived (5), embryonic stem cells and their derivatives (9), and induced pluripotent stem cells (iPSCs) (10,11). Nevertheless, ease of availability without moral and ethical strings and differentiation capacity to adopt desired phenotype are vital issues for the search of ideal donor cells for transplantation therapy. Given their association with the nervous system and capability of differentiation into the three neural lineages including neurons, oligodendrocytes and astrocytes, neural stem cells in particular are being considered as near-ideal cells for the treatment of stroke (3).

Experimental animal studies provide pre-clinical evidence regarding the safety and feasibility of the stem cell based reparative approach for ischemic stroke which has been vindicated during the clinical studies (12,13). The beneficial therapeutic outcome from the donor cells is considered as multifactorial that ranges from neurogenesis to the release of bioactive molecules as part of their paracrine activity to support the endogenous repair mechanisms in the damaged area of the brain (14,15). The donor cells repopulate the ischemic area and get integrated into the host circuit besides providing protection benefits to impart functional recovery (3,14,16). Despite encouraging results, multiple variables that are determinants of the success of cell therapy for stroke treatment remain less well-studied. These variables range from cell type selection to route of administration and optimum time duration after stroke to carry out stem cell transplantation to achieve best prognosis. The present study was aimed to assess the optimal time of NSC therapy post ischemic insult. Using rodent model of experimental ischemic stroke by middle-cerebral artery occlusion (MCAO), *in vitro* cultured NSCs were injected at stipulated time-points ranging from 1 hour to 7 days. The animals after their respective treatment on the stipulated time points were assessed for the neurological outcome, dUTP nick end labeling (TUNEL) assay and also the Caspase 3 activity to identify the apoptosis. Our results highlight the importance of early injection of the stem cells to curtail ischemic tissue injury to the brain during stroke.

Methods

The present study conformed to the Guideline for the Care and Use of Laboratory Animals and all the experimental animal procedures were performed strictly in accordance with protocol approved by Shiraz University of Medical Sciences, Iran. All surgical manipulations were carried out under general anesthesia.

Isolation of NSCs

NSCs were isolated from the ganglion eminences dissected from E14 (14-day-old) embryos of Sprague-Dawley rats using our standard protocol. Briefly, the heads of the embryos were separated and the brain tissue was dissected to separate cortices, midbrain and stria. The dissected tissue was transferred to the NSC culture media DMEM/F12 (Invitrogen Cat #10565018) supplemented with 2% B27 (Gibco Cat #17504044), 1% N2 (Invitrogen Cat #17502048, 10 ng/mL basic fibroblast growth factor (bFGF; Sigma Cat #F0291) and 20 ng/mL epidermal growth factor (EGF; Sigma E9644). The isolated tissues were mechanically dissociated and pipetted for reaching single cells to make a uniform suspension. The cells were seeded at density 50,000 cells/mL in culture dish at 37 ℃ and 5% CO_2. Neurospheres appeared by day 5 (17). For identification of NSCs, immunocytochemistry was performed using antibodies specific for Nestin (Abcam Cat #6142) and CD133 (Millipore; Cat# MAB4399) respectively.

Tri-lineage differentiation of NSCs

Single cell suspension of passage# 4 NSCs was prepared by treatment with 0.05% trypsin (Gibco Cat #25300054). The cells were later cultured on polyornithine coated plates (Sigma Cat #P3655) for 2 days. For induction of tri-lineage neural differentiation, 0.5% fetal bovine serum (FBS) (Gibco Cat #26140079) was added to the NSCs culture medium while concomitantly removing both bFGF and EGF. Three days later, the NSCs were differentiated into neurons, oligodendrocytes and astrocytes. To confirm the differentiation of the NSCs, immunocytochemistry was performed for β-tubulin III (neuron marker), glial fibrillary acidic protein (Gfap; an astrocyte marker) and Oligodendrocyte marker Olig2 as described earlier (18).

Immunocytochemistry for tri-lineages cells markers

Immunostaining of cells for specific markers was essentially carried out according to our standard protocols as described earlier (18). Briefly, the cells were cultured on glass slides and fixed with 4% paraformaldehyde for 20 minutes at 4 ℃. The cells were washed ×3 with phosphate buffered

saline (PBS) followed by incubation with respective primary antibody in PBS containing 0.3% triton and 5% goat serum, at room temperature for 1 hour. Primary antibodies used included anti tubulin-III (Promega Cat #G7121; 1:2,000), anti-Olig2 antibody (Millipore Cat# AB9610; 1:500) and anti-Gfap (Dako Cytomation Cat #Z0334; 1:500) for neurons, oligodendrocytes and astrocyte detection respectively. The cells were then washed ×3 with PBS and respective incubated with fluorescent-conjugated secondary antibodies for 45 minutes at room temperature. The nuclei were labeled with 4,6-diamino-2-phenylindole dihydrochloride (DAPI; Millipore Cat #S7113, 1:1,000) as described earlier (18). The samples were later fixed and visualized under fluorescence microscope (Olympus BX53 Japan) fitted with camera and software Cell-sens.

Experimental animal model of ischemic stroke and cell transplantation

The rodent experimental model ischemic stroke was developed in young (10–12 week old) male Sprague Dawley rats (n=120) each weighing 250-300 g by MCAO as described earlier (19). All the animals were allowed for free access to food and water before and after the surgical procedure. Briefly, the rats were anesthetized using Isoflurane (induction 5% and maintenance 1%). Following tracheal intubation and ventilation using Small Animal Ventilator (Harvard Model-683, USA), a vertical incision was made in the midline of the neck. The right common carotid, internal carotid and external carotid arteries were exposed and separated from vagus nerve. Two loose sutures were prepared below carotid bifurcation and external carotid was clamped, the silicone-coated nylon suture 4.0 was passed through a little incision in common carotid artery. After 30 minutes, the nylon suture was removed and the sutures were tightened up so that the blood could flow via external carotid artery by removing the clamp.

The animals were divided into 8 groups (n=15 animals/group) for their respective treatment. The sham group (G-1) did not receive any treatment whereas control group (G2) received 200 µL PBS. For experimental groups (EG) of animals, cell transplantation was carried out at 1 hour (EG1), 12 hours (EG2), 1 day (EG3), 3 days (EG4), 5 days (EG5) and 7 days (EG6) after MCAO respectively. For stereotactic injection of NSCs at stipulated time-points after induction of MCAO, the animals were anesthetized with Isoflurane (induction 5% and maintenance 1%) and then fixed to the stereotactical frame. A total of 200,000 cells suspended in 200 µL PBS were injected into right lateral ventricle at anteroposterior (AP) =–0.12 mm, mediolateral (ML) = 1.6 mm and dorso-ventricular (DV) =4.3 mm. The animals were allowed to recover and buprenex (0.1 mg/kg b.i.d) was administered for 24 h to alleviate pain.

Histological studies

After 28 days of their respective treatment, the animals were euthanized with deep anesthesia and perfused with normal saline followed by Paraformaldehyde 4%. The specimens of brain were prepared for cryosections (at a thickness of 10 µm) and then the sections were mounted on silicon pre-coated slides. The specimens were stained with Hematoxylin & Eosin (H&E) to visualize the architecture.

Caspase-3 and TUNEL assays

TUNEL assay was performed to determine the number of apoptotic cells in the damaged area. The sections were stained by using terminal deoxynucleotidyl transferase-mediated (TUNEL) *in situ* Apoptosis Detection Kit (Chemicon International, Inc., USA).The images were taken by fluorescent microscopy (Olympus BX53, Tokyo, Japan).

Neurological function assessment

The neurological examinations of the animals were performed every 2 days for all rats during 28 days of experiment after their respective treatment. The neurological examination was scored on 0–5 score scale as described earlier (19). The scores criteria was: No neurological deficit (score =0); failure to extend the left forepaw completely to reflect mild focal neurological deficit (score =1); circling to the left to reflect a moderate focal neurological deficit (score =2); falling to the left indicating a sever focal neurological deficit (score =3); no spontaneous walking and decreased level of consciousness (score =4) and death due to brain ischemia (score =5).

Statistical analysis

Data were presented as mean ± SD. For quantitative analysis, data was analyzed with student *t*-test and one-way ANOVA with post hoc analysis using SPSS 16.00. A value of $P<0.05$ was considered as statistically significant.

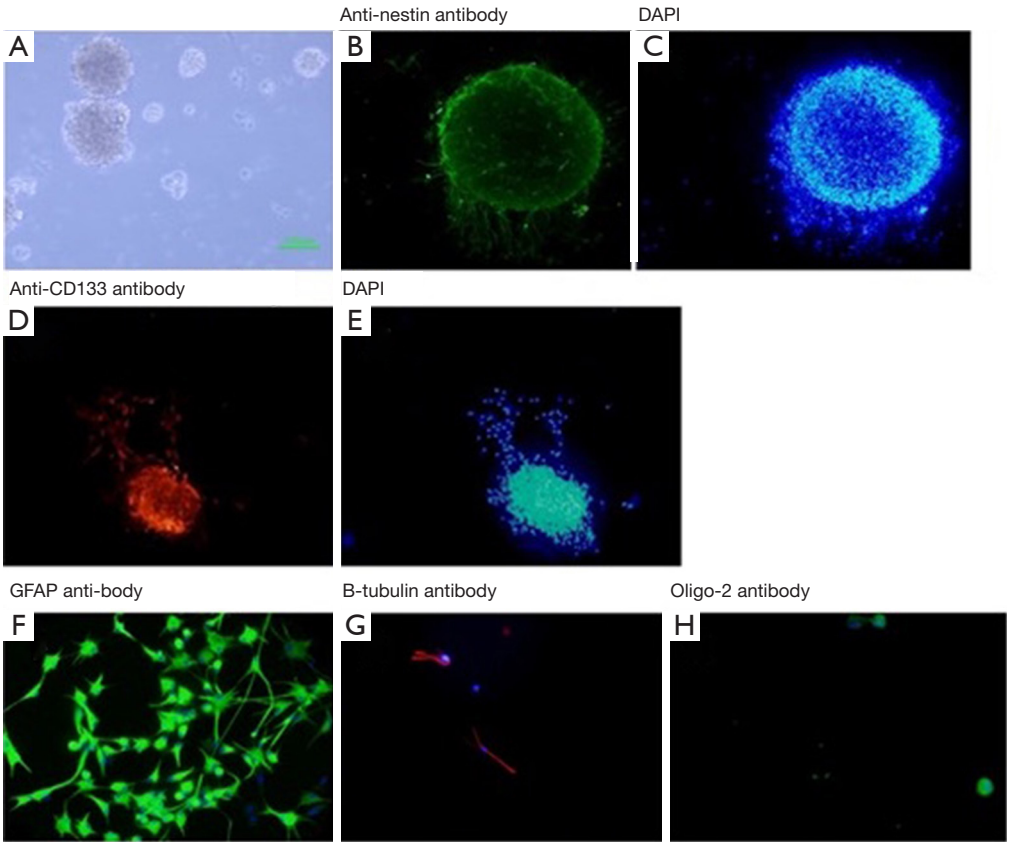

Figure 1 Cell culture and immunocytochemistry of neural stem cells (NSCs). The cells were isolated from 14 days embryo rat ganglion eminence and subsequently prompted for trilineage differentiation. (A) Neural stem cell cultured *in vitro* to form neutrosphereson day 5 after isolation under phase contrast microscopy (×20). (B-E) Immunostaining of the cultured NSCs for (B) nestin and (D) CD133 expression (NSCs markers) using their respective specific antibodies. (C&E) The nuclei were visualized using DAPI. (F-H) Immunostaining of differentiated NSCs for expression of specific markers: (F) Gfap (for astrocytes), (G) β-tubulin (for neurons) and (H) Olig-2 (for oligodendrocyte) using their respective specific antibodies.

Results

NSCs expansion and characterization

The NSCs were successfully isolated from rat embryos and cultured *in vitro* under well-defined culture conditions. On day 5 in culture after isolation, NSCs were observed to form neurospheres (*Figure 1A*). Immunocytochemistry of the isolated neural cells that formed the neurospheres stained positively for the expression of specific markers including Nestin (*Figure 1B,C*) and CD133 antibody (*Figure 1D,E*). Removal of growth factors bFGF and EGF and supplementation of the culture medium with 5% FBS promoted trilineage neural differentiation of the NSCs. Immunofluorescence staining using β-tubulin-III antibody (for neuron detection), Gfap (for astrocyte detection) and Olig-2 (for Oligodendrocyte detection) showed that 17.43%±3.02% of the cultured NSCs were positive for β-tubulin-III (*Figure 1F*) while 70.50%±6.29% of the differentiated cells were positive for Gfap (*Figure 1G*) and 8.94%±1.32% cells were positive for Olig-2 (*Figure 1H*).

Histological studies

At stipulated time-points, the animals were deeply anesthetized and perfused with normal saline followed by 4% Paraformaldehyde. The cryosection of 10 µm thickness were cut and selected with 1 mm interval and the first one was 1 mm posterior to frontal lobe. The damaged area

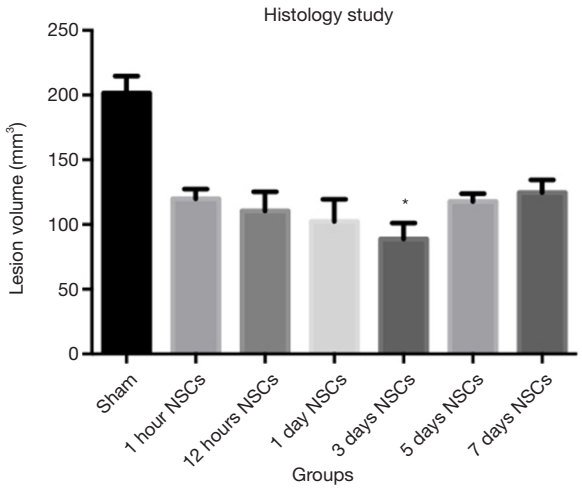

Figure 2 Histology study: the quantification for the H&E staining for detecting the infarct area volume. This graph indicates that the group which received NSCs 3 days after stroke had less infarct volume. *P<0.05.

was determined by ischemic sings containing eosinophilic cytoplasm and pyknotic nuclei. The volume area of sham group was 191.39±12.53 m^3 and the group which received NSCs 3 days after ischemia had the least volume of damaged area 68.13±4.93 m^3. There was a significant difference between the 3 days after ischemia receiving NSCs and the rest groups Tukey's multiple comparison test and also all groups' neurological outcomes had significant differences in comparison to sham group (*Figures 2*,*3*). These data confirm that the NSCs transplantation 3 days after stroke could promote histologically improvement of the brain.

Apoptosis evaluation

Caspase 3 is an integral member of the apoptosis cascade (20). The caspase-3 activity assay in different treatment groups showed least amount of caspase-3 activity in the day 3 treated animal group after stroke as compared to all other groups (P<0.05; *Figure 4*). These data were duly

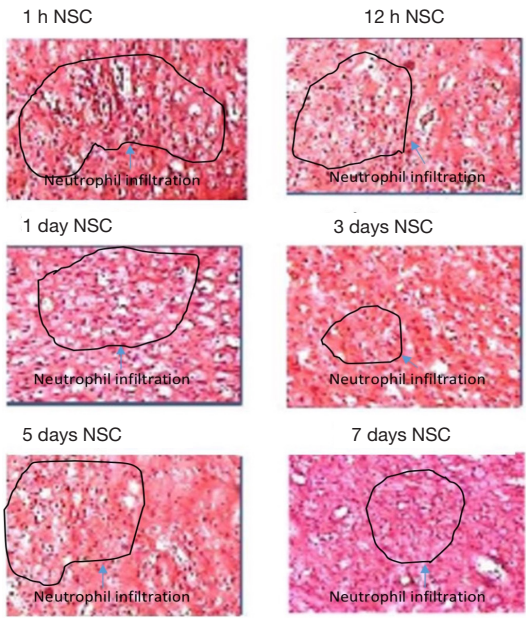

Figure 3 Histochemical studies using H&E staining from different treatment groups showing development of ischemic area infiltrated by inflammatory cells at different time points after stroke and cell therapy (×20). The arrows and also the lines show neutrophil infiltration as a strong marker for inflammation and also the ischemic area.

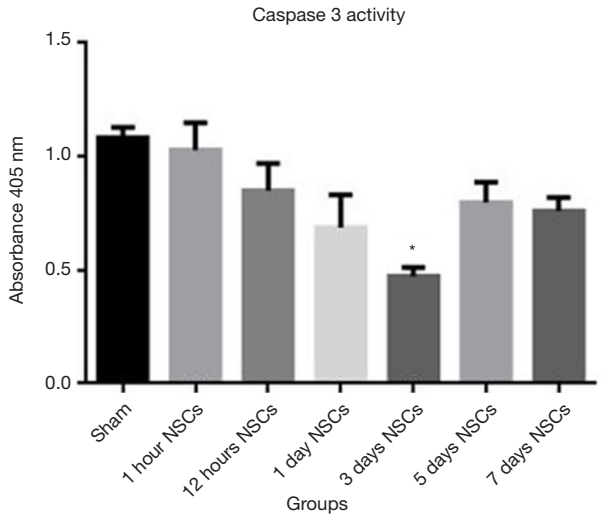

Figure 4 Caspase 3 activity assay. The assay was performed using commercially available kit (Abcam ab39401) and the assay samples were assessed at 405 nm. The lower caspase-3 activity indicated small level of apoptotic activity in the samples. Caspase-3 activity was significantly lower in samples from day 3 treatment group of animals as compared to the sham operated controls (*P<0.05).

supported by TUNEL assay which showed that TUNEL positive cells were least in the day 3 treatment animal groups after stroke injury as compared to all other groups (*Figure 5*). The highest level of TUNEL positive cells was observed in the animals which did not receive NSCs after stroke injury. The number of TUNEL positive cells was 12.92%±2.26% in that group and there is a significant difference between that group and the others (P<0.05). Histological studies after H&E staining showed significant infiltration of inflammatory cells in the ischemic zone which was significantly reduced in the day 3 animal groups as compared to the other treatment groups (*Figure 3*).

Neurological examination

All of the groups were examined during the experiment for the stipulated time duration of 28 days. The animals in each group completed full length of the experiment and there was no death related with the cell treatment. Neurological examination scores were analyzed with Sidak's multiple comparison tests. There was significant difference between

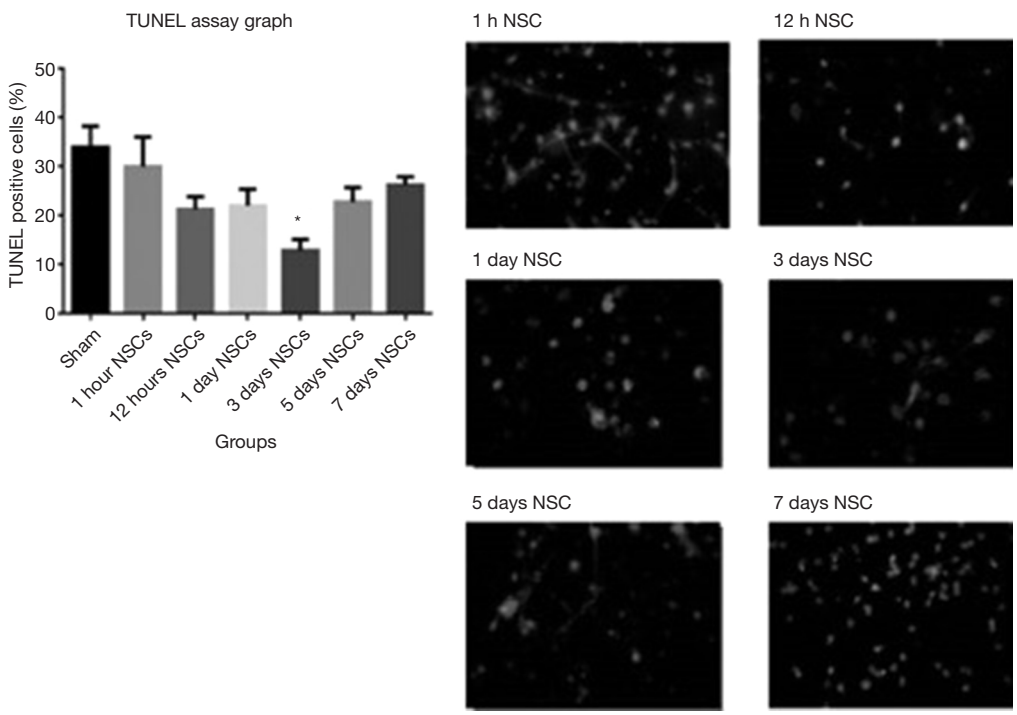

Figure 5 TUNEL assay for cell apoptosis. The assay was performed with TUNEL assay kit (Chemicon International, Inc., USA) using histological tissue sections from different treatment groups of animals on day 28 after their respective treatment. The number of TUNEL positive cells was significantly lower in day 3 cell treated animals group (*P<0.05 *vs.* all other treatment groups) as compared to the control.

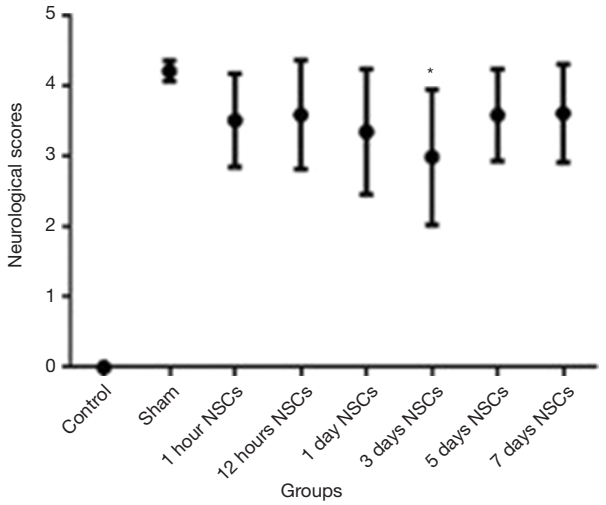

Figure 6 Assessment of neurological performance in different animal groups. Neurological performance significantly preserved in day 3 cell treatment group of animals. The data was analyzed by Prism 6.00 software. *P<0.05.

the mean of the 3 days NSCs injection neurological scores (2.99±0.19) with sham operated group and all the other cell treatment groups (P<0.05 *vs.* all other treatment groups). Also, there were significant differences (P<0.05) between the sham group and all cell therapy groups (*Figure 6*).

The final goal of every treatment on stroke could be described as enhancing neurological function. According to these data, all cell therapy groups could perform better than sham operated group. In addition, a significant difference is observable in all cell therapy groups which means that different cell therapy protocol could modify the outcome of the treatment. The graph in *Figure 6* shows the NSC transplantation 3 days after stroke could be considered as an effective way to optimize the neurological performance.

Discussion

The main findings of our study are that the: (I) NSCs successfully attenuated infarct size expansion and provided best neurological outcome; (II) optimum time for stem cell therapy was 3 days post ischemic stroke; and (III) NSC transplantation prevented host cell apoptosis as one of the possible underlying mechanisms to attenuate infract size expansion. It is noteworthy that attenuated infarct size, reduction in TUNEL positivity and decreased caspase levels corresponded well with day 3 of NSC transplantation.

The brain being a highly perfused organ in the body utilizes more than 20% of the total oxygen and hence the cells therein are exceedingly sensitive to ischemia (21). Subsequent to the ischemia, as the events progress, inflammatory response causes loss of neural cells both in the center of the ischemic zone, mainly by necrosis, as well as in the penumbra, mostly by apoptosis (22,23). During acute phase, elevated levels of the otherwise lowly expressed plethora of molecules such as intercellular adhesion molecule-1 (ICAM-1), P-selectin and matrix metalloproteinases are positively correlated with clinical worsening of stroke patients thus leading to poor prognosis (24,25). Incidentally, polymorphism in MMP-9 gene and the expression of level of MMP-9 has been attributed to the risk of stroke and as a marker for the loss of blood brain barrier, edema and intensity of the inflammatory response following ischemic stroke respectively (26,27). Similarly, the elevated levels of pro-inflammatory cytokines such as tumor necrosis factor-1α (TNF-1α), interleukins (IL) IL-1, IL-6 and IL-10 and the chemokines including macrophage inflammatory protein-1α (MCP-1α) and fractalkine affect higher infarct size following ischemic insult (28,29). The end-result of these molecular events is high level infiltration of the inflammatory cells that renders the ischemic area and its penumbra non-conducive for physiological functioning of the brain cells. Unless a therapeutic intervention prevents these nocuous events, massive death of the brain cells ensues owing to the hostile microenvironment in the ischemic area. The combined effect of the cellular and molecular events also significantly impacts the survival of donor cells at the site of the cell graft that remains a serious concern in regenerative medicine for stroke. NSCs are more prone to succumb to ischemic injury when engrafted for the treatment of ischemic stroke. As little as 0.09% survival rate of the donor cells have been reported in the experimental model of ischemic stroke (30). Although the transplanted cells that survived the initial surge of cell death manage to enter cell cycle, the rate of proliferation was meager and only 0.23% cells were observed in the center and peri-infarct regions on day 10 after intravenous delivery (30). Various strategies have been adopted to enhance the donor cells survival post-engraftment including ischemic preconditioning, pre-treatment with pro-survival pharmacological compounds, growth factors, cytokines and by genetic modification (31-34). Additionally, genetic manipulation of stem cells with hypoxia induced microRNAs (miRS) in general and miR-210 in particular have shown encouraging results in terms of improved cell survival (35). Besides other factors, time of

cell engraftment after ischemic injury is considered as an important determinant of donor cell survival. If the cells are transplanted too early during the acute phase of ischemia, the cascade of inflammatory response turns the host tissue environment unfavorable for their survival. On the contrary, if cell transplantation is deferred until the inflammatory response is subsided; it would not be possible for the donor cells to revert the scarring process that would have set-in by that time. Furthermore, brain tissue plasticity would be diminished and apoptosis in second injury could make neural regeneration harder than subacute time. Hence, the time of cellular intervention post ischemic insult is integral to the accomplishment of the desired prognosis. Although methodical and in-depth studies to ascertain optimal intermission between stroke to cell transplantation are still wanting in the literature, various research groups have performed cell transplantation from hours to weeks after ischemic stroke (36,37). Our study was aimed to ascertain the optimal time for cell therapy after stroke in terms of attenuation of cell apoptosis in the host tissue and overall improved neurological performance. We observed that the rate of TUNEL positive cells was significantly higher in the earlier time-points of NSC treatment animal group as compared with the day 3 treatment animal groups. These observations were supported by the attenuated brain lesion volume in day 3 treatment group of animals as compared to the other treatment groups. It would have been interesting to determine the rate of donor cell survival and discriminate the TUNEL positive cells as host and the donor cells which are the two main limitations of our study. Our data is compatible with the previously published studies which demonstrated that the optimum time for intervention with NSCs was 3 days after ischemic stroke and it is related to diminished inflammatory response after the acute phase and the peak of post-ischemic apoptotic surge (29). A recent study involving intra-arterial delivery of mesenchymal stem cells in a rodent model of MCAO showed that time of cellular intervention drastically impacted the donor cell distribution and functional recovery in the experimental group (38). The authors observed motor functional recovery in the animals which received cell transplantation therapy on day 4 as compared to those treated on day 1 and day 7 after experimental ischemic stroke. Moreover, the rate of cell transplantation related mortality amongst the day 1 (10.1%) and day 4 (19.2%) treatment groups was significantly low as compared to the day 7 treated animals (30.8%). The same group of researchers reported day 3 as the optimal time for intra-parenchymal transplantation of NSCs (15). In both the studies, the beneficial effect of cell transplantation was attributed to multiple factors including the release of trophic factors, especially vascular endothelial growth factor (VEGF) and angiogenesis besides timing of cell injection. Paracrine release of neuroprotective, angiogenic and pro-survival trophic factors has also been reported by many other research groups as central to the therapeutic benefits of cell transplantation therapy for stroke albeit with variation in the expression profile of the trophic factors being specific to the cell type used engraftment (8,9,15,39). The bioactive molecules released by the transplanted cells not only promote their own survival but also participate in the endogenous repair mechanisms by augmenting survival of the host cells in the vicinity. These cellular events lead to attenuated infarct size expansion. Although neuronal differentiation of the transplanted cells has also been reported but the rate of cell differentiation is insufficient to justify its serious contribution as a major contributing factor to the overall functional benefits of cell therapy. Therefore, besides choice of ideal donor cell type and appropriate pro-neural differentiation cues, pro-survival strategies would be pivotal to enhance the probability and rate of differentiation of the donor cells. For that matter, timing of cell transplantation would be a fundamental consideration to ensure that the transplanted cells escape the primary inflammatory response due to ischemia.

In conclusion, our data shows that day 3 after ischemic stroke is optimal for cellular intervention in terms of reduced apoptosis and quelling infarction size expansion and preservation of neurological functions. However, more systematic experimental studies would be required wherein donor cell survival and mechanistic molecular insight into the beneficial outcome after cell therapy should be carried out to define parameters such as ideal cell type, route of administration and time of cell delivery before routine application in the clinics.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

Ethical Statement: The study was approved by Institutional Ethics Committee of Shiraz University of Medical Sciences,

Iran (No. 7643).

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