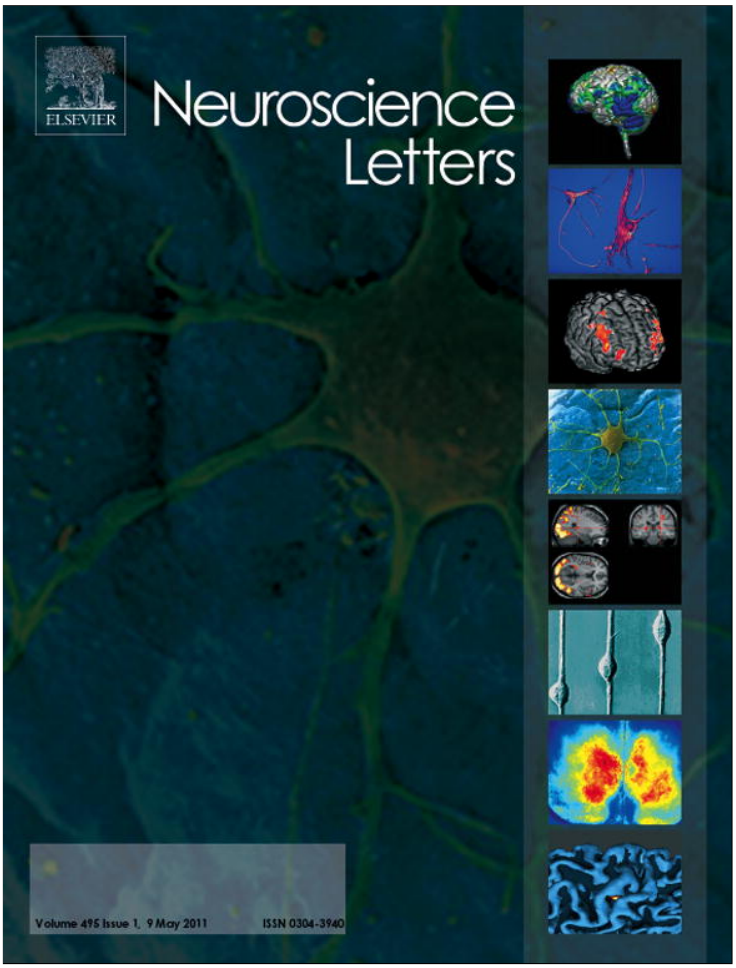

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Investigating the effects of adult neural stem cell transplantation by lumbar puncture in transient cerebral ischemia

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ABSTRACT

Stem cells have the ability to self renew and are therefore a good source for cell therapy following ischemia. In this study, we transplanted adult rat neural stem cells (NSCs) by lumbar puncture (LP) to investigate whether these cells can migrate and differentiate into neurons or glial cells, thereby improving functional outcome in cerebral ischemia. Transient ischemia was induced in adult rats ($n = 16$) for 1 h. Three days after the induction of ischemia, NSCs obtained from the subventricular zone of adult rats were injected into ischemic animals ($n = 8$) by LP at the level of L6–S1. Improved recovery of the coordination of movement on the 1st, 7th, 14th, 21st and 28th days after the injury was examined by the Rotarod test and compared with non-transplanted ischemic animals ($n = 8$). The presence of NSCs in the brain tissue of the animals was examined by immunohistofluorscence and immunohistochemical techniques. The coordination of movement in ischemic animals that received neural stem cells was improved significantly ($P < 0.05$) compared with untreated ischemic animals. Cells labeled with PKH26 were observed in the ischemic area of brain tissue sections. The alkaline phosphatase test and immunohistochemical techniques demonstrated a gathering of NSCs in the lateral ventricle. A number of cells which expressed neuronal and astrocytic cell markers had migrated from the lateral ventricle to the subjacent brain parenchyma. NSCs injected by LP were able to migrate to the ischemic tissue and differentiate into neural-like cells. These differentiated cells may have improved the coordination in movement in the ischemic animals injected with NSCs.

Ischemia can cause damage to the brain that is associated with molecular and biological changes leading to the induction of cell death [12] and neurological symptoms [6]. NSCs are able to proliferate in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF), and when these factors are removed from the culture medium, they differentiate into neurons and glial cells of the CNS [17,19]. Neurons that are generated from NSCs also have the ability to form functional synapses in vitro [20] and in vivo [1].

It has been shown that NSCs transplanted into the brain ventricles form clusters and some traverse the ventricular wall to the subventricular zone (SVZ). After inducing focal transient ischemia, these cells migrate to the striatum, particularly to the periphery of the portion that was damaged by the ischemia [9].

Transplantation of neural progenitor cells by direct injection to ischemic models causes damage to the brain parenchyma by the injecting needle and limits the migration of the cells [5,18,21]. Therefore, transplantation of NSCs into the cerebrospinal fluid (CSF) by lumbar puncture LP is an attractive choice in relation to the other methods because of its minimal state of injury, low cost and simplicity [11].

Many studies have demonstrated the survival and migration of the NSCs in replying to the focal injury [15,16]. Previous studies have suggested that NSCs have the potential to differentiate into appropriate neurons to form a functional neuronal circuitry. Therefore, NSCs could be used as a valuable source for cell therapy by transplantation in any neurological injuries [14].

The purpose of this study was to transplant NSCs using lumbar puncture, which is a safe and cheap method, into rats following transient ischemia to investigate whether these cells can migrate and differentiate into neurons or glial cells, and thereby improve the coordination of movement.

Two-month-old male rats were deeply anesthetized with chloral hydrate (400 mg/kg, I.P.) and sacrificed by cervical dislocation. The animals were decapitated and the brains were excised under sterile conditions [3]. The SVZ was dissected and cut into small

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pieces. Tissue specimens were transferred into 0.25% Trypsin/EDTA. After 30 min, the trypsin was inactivated by fetal bovine serum (FBS, Gibco, Australia). The tissue was mechanically dissociated into a cell suspension with a fire-polished Pasteur pipette. After centrifugation, the cells were plated in uncoated tissue culture flasks containing DMEM (Sigma) with N2 supplement, 20 ng/ml (Gibco, Australia) EGF, 20 ng/ml FGF2 (Sigma–Aldrich, MO, USA) and penicillin/streptomycin (100 U/ml, 100 μg/ml). Every two days, half of the culture medium was replaced by fresh medium. After 3 days, some neurospheres appeared. The neurospheres were passaged weekly.

Neurospheres were dissociated to single cells and plated into 96-well plates coated with poly-D-lysine containing DMEM/F12 with N2 supplement and 10% FBS. The cultures were grown for 7 days, by the time which morphology of the cells changed. After 7 days, they were fixed with 4% paraformaldehyde (PFA). The cultures were then washed three times with PBS. After treating with 0.3% Triton X-100 for 20 min, cultures were blocked with 5% normal goat serum in PBS and 0.3% Triton X-100 for 1 h at room temperature and exposed to the following primary antibodies: anti β-tubulin 3 (1:200; Abcam), anti S100 (1:200; Abcam) and anti CNPase (1:100; Abcam), and incubated at 4 °C overnight. The cultures were washed three times with PBS and treated with goat anti-mouse FITC conjugated IgG (1:50; Chemicon) secondary antibody at room temperature for 1 h. They were washed three times with PBS and prepared for observation under a fluorescent microscope (Olympus IX71, Olympus, Japan) equipped with a digital camera (DP71, Olympus, Japan).

Male Wistar rats weighing 280–320 g were anesthetized with chloral hydrate (400 mg/kg I.P.) and an operative field was shaved. A skin incision was made at the midline of the neck. The neck fascia and muscles were dissected to gain access to the right common carotid artery (CCA). The CCA was temporarily ligated and the internal carotid artery (ICA) was clamped by a microsurgery clamp. A cut through the external carotid artery (ECA) was made and a 20-mm-long 3–0 monofilament nylon suture with one end sealed by flame and coated with poly-D-lysine was inserted from the ECA to the clamped right ICA. The clamp was removed and the nylon suture was allowed to continue to occlude the right middle cerebral artery (MCA) [13]. After 60 min, the nylon suture was removed to allow reperfusion. During the operation, rectal temperature was monitored and kept at 37 °C using a heating lamp. The sham-operated animals ($n = 8$) underwent the same surgery except that the suture was not inserted. After 24 h, the animals were examined for neurological signs. The animals with a contralateral rotation and forelimb positive test were selected. Induction of ischemia was verified by the observation of necrosis in the cortex and caudate nucleus and putamen regions at the end of the experiments.

Three days after the induction of ischemia, eight out of 16 ischemic animals were anesthetized with chloral hydrate (400 mg/kg) and placed on an operating table [2]. The animal's forelimbs were extended towards the front and fixed with tape. The hindlimbs were left to hang off the table in a way that trunk and hind limbs made right angle to each other. A longitudinal incision was made over the L5–S1 spinous processes and the skin was retracted. A neonatal LP needle (24G) was advanced into the spinal canal at the level of L5–L6 or L6–S1. The correct placement of the needle was confirmed by two signs: (1) the tail flick, and (2) the presence of CSF in the needle hub. NSCs were labeled by PKH26 and 1×10^7 cells in 50 μl PBS were injected into CSF. After recovery, the animals were returned to their cages and immunosupressed by I.P. administration of cyclosporine A (10 mg/kg) (Novartis, Sweden), 24 h before transplantation and daily thereafter. The animals were divided into five groups: (1) control group that did not receive any intervention ($n = 8$), (2) sham operated group in which only the skin of the neck was incised and then sutured ($n = 8$), (3) ischemic group ($n = 8$), (4) ischemic group that received NSCs by LP ($n = 8$) and (5) vehicle group that received the vehicle instead of NSCs ($n = 5$).

A behavioral test was performed one day before the induction of ischemia, and at the 1st, 2nd, 3rd, and 4th week after the induction of ischemia by a person who was blinded to the experimental groups [4]. The animals were placed on an accelerating Rotarod cylinder and the time that they remained on the Rotarod was recorded. The test was stopped if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions without attempting to run. The maximum time that each animal remained on the device was recorded.

After the last Rotarod test, the animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA) in PBS, pH 7.4. The brains were removed carefully and immersed in 30% sucrose in PBS for 24 h at 4 °C. Cryostat sections (20 μm) were cut and mounted onto gelatin-coated slides and stored at −70 °C. The tissues were incubated in 5% normal goat serum containing 0.3% Triton X-100 for 45 min. Then they were incubated with the following primary antibodies: anti β-tubulin 3 (1:200; Abcam) and anti S100 (1:200; Abcam) at 4 °C overnight. The sections were washed three times with PBS and treated with a goat anti mouse FITC conjugated IgG (1:50; Chemicon) secondary antibody at room temperature for 1 h. The slides were washed three times with PBS and prepared for observation under a fluorescent microscope (Olympus IX70, Olympus, Japan).

Brain tissues were incubated at 60 °C for 60 min to inactivate the endogenous peroxidase. They were stained with an alkaline phosphatase kit (Sigma–Aldrich, MO, USA) according to the manufacturer's protocol for the detection of alkaline phosphate positive cells. The tissues were examined under a light microscope (Nikon Ts100, Nikon, Japan).

All the data are presented as the mean ± SEM. The *t*-test was used to compare differences between two groups. SPSS software version 11.5 for Windows was used for statistical analysis. A difference of $P < 0.05$ was considered significant.

Following three days culture, cells which were obtained from the SVZ region of rat brains in medium containing mitogenic factors (EGF and FGF) formed a number of floating clustered neurospheres. Dissociated neurospheres, which were cultured in poly-D-lysine coated 96 well plates for 7 days, attached to the bottom of the plates and exhibited neural morphology. Some of these differentiated cells were positive for anti β-tubulin 3, anti S100 and anti CNPase markers, which are representative of neuronal, astrocytic and oligodendrocytic cells, respectively.

To determine whether the transplantation of NSCs by LP improved the coordination of movement after the induction of ischemia, the Rotarod test was performed. The coordination of movement of the NSC receiving ischemic animals was significantly higher ($P < 0.05$) than the ischemic animals which did not receive NSCs (Fig. 1). Following the injection of NSCs, the coordination of movement increased with the time, and on the day 28th, it was statistically significant ($P < 0.05$). The average score of the coordination of movement was 43.9 ± 4.06 and 53.25 ± 5.43 for ischemic animals and ischemic animals that received NSCs, respectively ($P < 0.05$).

After the injection of PKH26-treated NSCs into the subarachnoid space of ischemic animals via LP, these cells were found in the brain ventricles freely and attached to the ventricular wall, and some had migrated to the subependymal zone (Fig. 2). We could also demonstrate some β-tubulin 3+/PKH26+ cells at the periphery of the ischemic area (Fig. 3). PKH26+ cells that were attached to the ventricular wall highly expressed the S100 marker, and a number of cells that migrated to the subependymal layer also expressed the S100 marker (Fig. 4). A number of transplanted NSCs stained with alkaline phosphatase had migrated to the subependymal zone (Fig. 5).

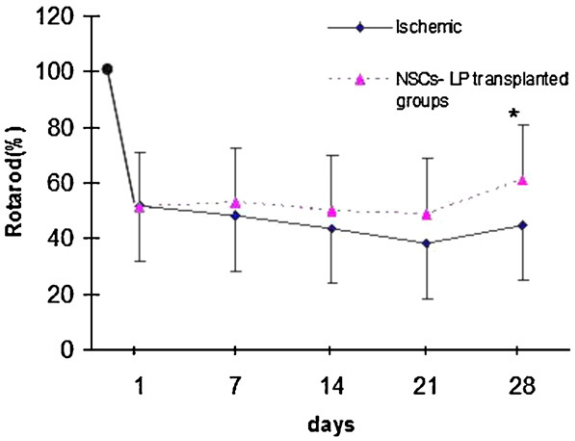

Fig. 1. Recovery of the coordination of movement in ischemic and non-ischemic animals. This figure shows that the coordination of movement was higher and significant in the ischemic animals that received NSCs than those that did not receive these cells at 28 days after ischemia.

In this study, we were able to demonstrate the potential of NSCs in the repair of focal cerebral ischemia in rats. We showed that NSCs which were transplanted by lumbar puncture had the ability to migrate to the ventricular system and then to the injured area, differentiating into phenotypic neural cells. The first attempts at transplantation of NSCs in situ led to neurological defects and was found to produce more injury during the process of injection. Therefore, other methods of transplantations such as intravenous [10], intracisternal [7] and intra-arterial [8] routes of delivery were developed for the therapy of neurological disorders. In this study, we insisted on a safe method of NSC injection into the animals with the cerebral ischemic model of injury with minimum injury to the cerebral tissue in the process of injection. There are only a few studies in which the injection of NSCs into ischemic animals was performed by LP. It has been shown in spinal cord injury models that LP injected neural progenitor cells, can migrate to the site of injury and differentiate into the type of cells that are situated in the region [11]. Lepore et al. [11] also demonstrated the migration of injected cells to the ventricular system by using the alkaline phosphatase staining technique. In our study, the alkaline phosphatase staining technique showed that, after the transplantation of NSCs by LP to ischemic rats, the migrated cells were observed as a gathering of cells at the margins of the ischemic ventricle which expressed neuronal and glial markers. We suggest that the gathering of the migrated cells in the ventricle of the ischemic hemisphere, in contrast to the intact hemisphere, could be under the influence of factors released from the cells in the ischemic area that attracted the injected cells to the area of injured brain tissue. These fac-

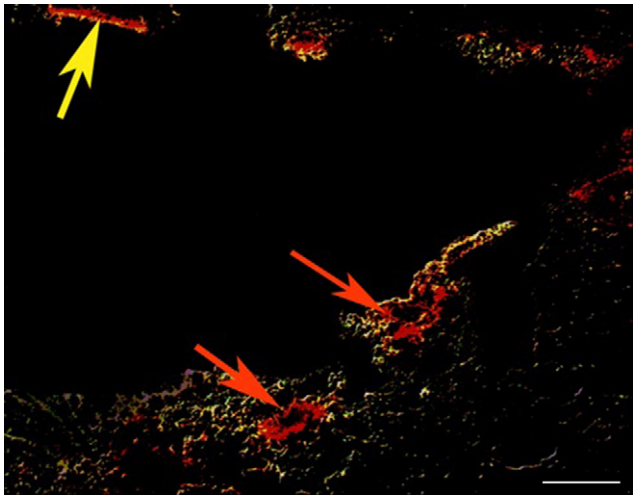

Fig. 2. Migration of NSCs to the ventricle and subependymal zone in ischemic animals. The image shows the appearance of cells that express the PKH26 marker in the ventricular wall (red stained cells showed by yellow arrow) and subependymal zone (showed by red arrows) of ischemic animals one week after NSC injection by LP (bar 200 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

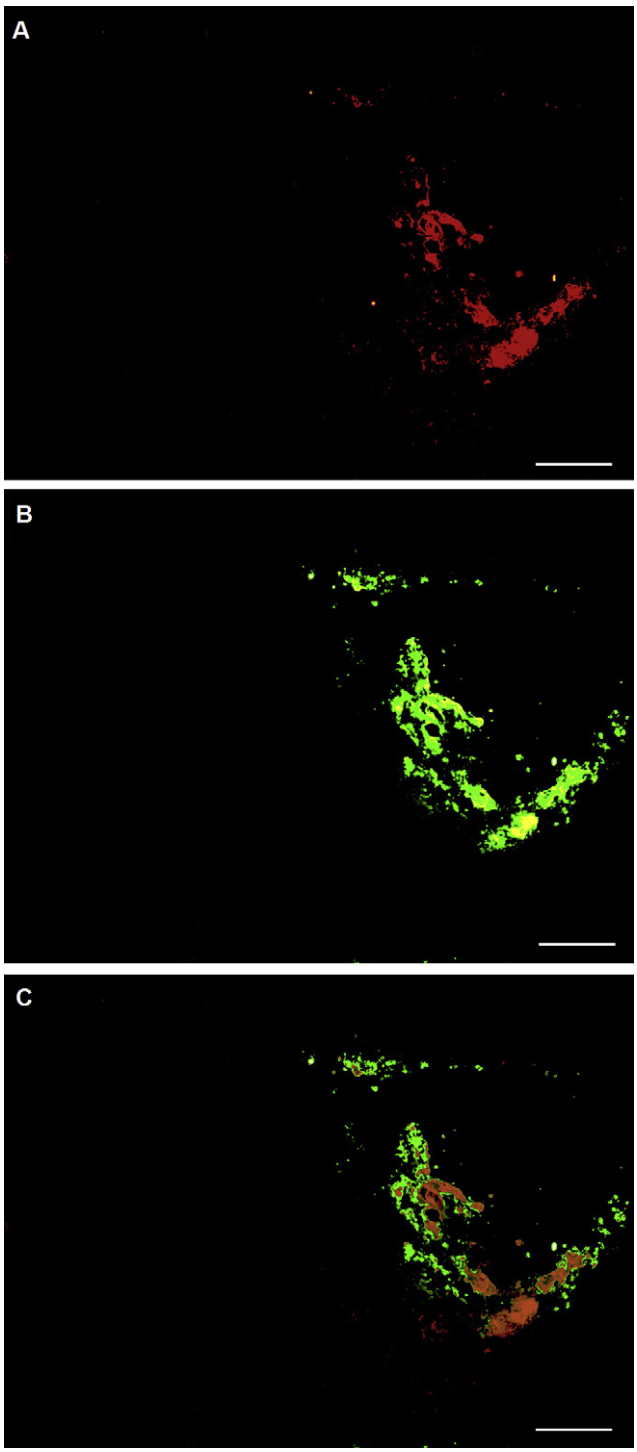

Fig. 3. (A) Appearance of differentiated NSCs as neurons in the ventricular wall and subependymal zone in ischemic animals. Transplanted NSCs stained with PKH26 seen in the ventricular wall. (B) Transplanted NSCs in the ventricular wall show high expression of β-tubulin 3. (C) A merged image of (A) and (B) (bar 100 μm).

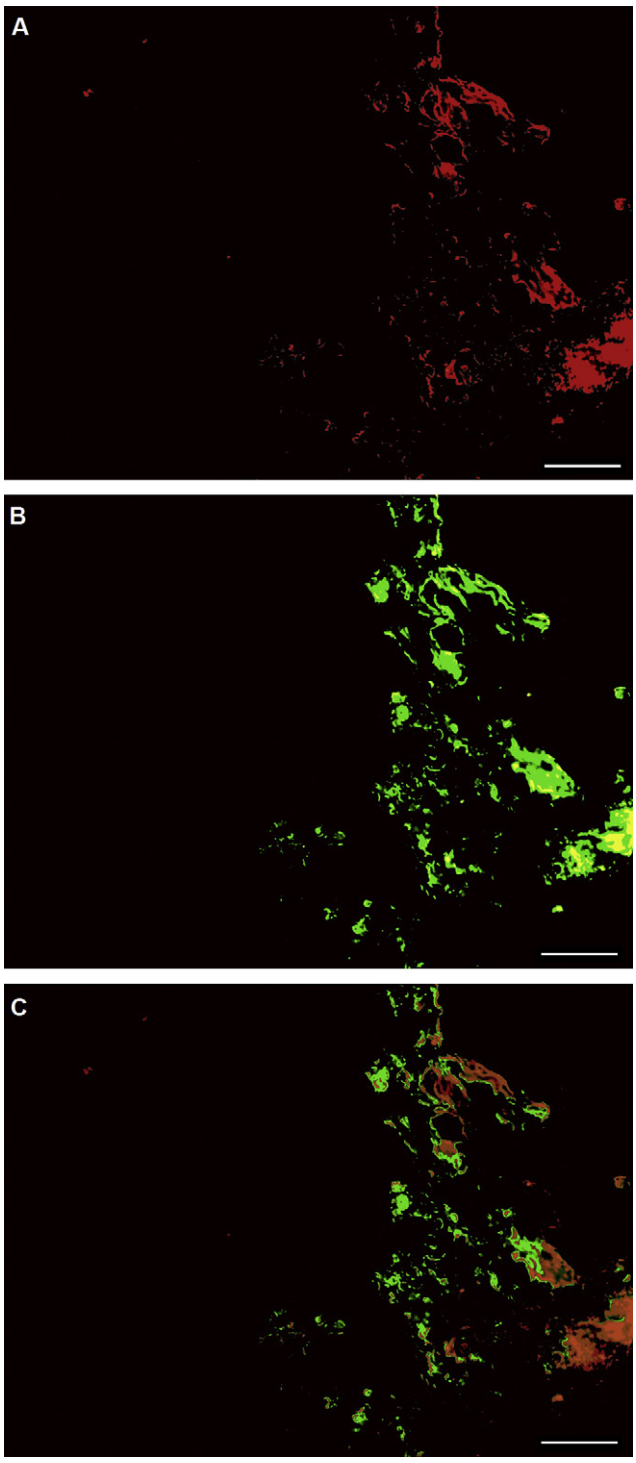

Fig. 4. (A) Appearance of differentiated NSCs as astrocytes in the ventricular wall and subependymal zone in ischemic animals. Transplanted NSCs stained with PKH26 are seen in the ventricular wall and subependymal zone. (B) Transplanted NSCs in the ventricular wall and subependymal zone show expression of the S100 marker. (C) A merged image of (A) and (B) (bar 160 μm).

tors should be released in the cerebrospinal fluid as well as in the blood, and the injected cells could be able to migrate along this chemoattractant gradient to the ventricle of the ischemic hemisphere and pass through the ependymal layer and into the brain tissue. Intraventricularly injected NSCs, in focal ischemic models of injury, have been seen to migrate through the ventricular wall to the SVZ and then to the area close to the striatum and injured brain

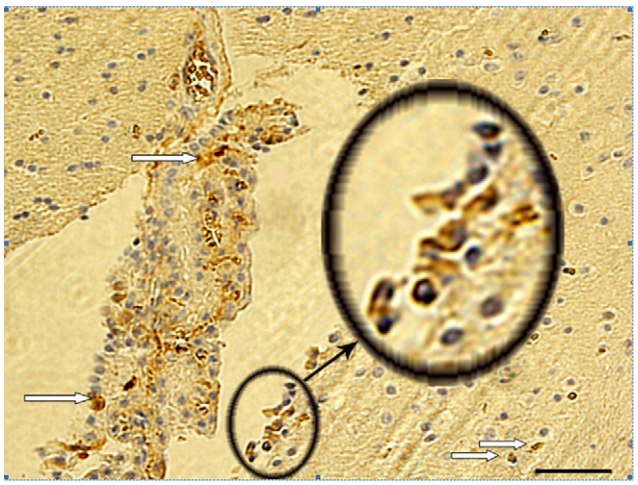

Fig. 5. Appearance of alkaline phosphatase treated NSCs in the ventricular wall and subependymal zone in ischemic animals. In this image, it seems that cells stained with alkaline phosphatase (brown cells) pass through ependymal layer to the subepnedymal zone and brain parenchyma. The inset shows a higher magnification of the ependymal layer and the abovementioned cells (bar 200 μm).

tissue. These migrated cells differentiated into the type of cells that existed in the area of injured brain tissue [9]. In our study, in addition to the demonstration of migrating and differentiating NSCs, we could also provide valuable information about the coordination of movement in animals which received NSCs. Intraventricular [9] and intracisternal [7] methods of NSC injection are invasive and difficult methods that could damage the brain tissue and floor of the fourth ventricle, but the LP method that we used is a simple, inexpensive and harmless method that can be used in other animals and in humans. Our unpublished data have shown the LP route of delivery as a safe method of transplantation of autologous bone marrow stem cells in the repair of spinal cord injured (SCI) patients.

We demonstrated that the NSCs that were injected into the cerebrospinal fluid of ischemic rats by lumbar puncture migrated to the injured tissue of the cerebral hemisphere and differentiated to neuronal and glial cells and therefore may lead to better coordination of movement. This effective and harmless method of cell transplantation both reduced the injury to the nervous system and caused the survival of injected cells leading to a better recovery of nervous system disorders. In this study, we demonstrated that the injection of NSCs by lumbar puncture may be a suitable method for treating neurological disorders.

Acknowledgement

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References

[1] J.M. Auerbach, M.V. Eiden, R.D.G. McKay, Transplanted CNS stem cells from functional synapses in vivo, Eur. J. Neurosci. 12 (2000) 1696–1704.
[2] A. Bakshi, C. Hunter, S. Swanger, A. Lepore, I. Fischer, Minimally invasive delivery of stem cells for spinal cord injury: advantages of the lumbar puncture technique, J. Neurosurg. (Spin 1) 3 (2004) 330–337.
[3] G.T. Gobbel, S.J. Choi, S. Beier, A. Niranjan, Long-term cultivation of multipotent neural stem cells from adult rat subependyma, J. Brain Res. 980 (2003) 221–232.
[4] J.R. Hamm, R.B. Pike, M.D. O'Dell, G.B. Lyethe, W.L. Jenkins, The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury, J. Neurotruma 11 (1994) 187–196.
[5] H. Hodges, P. Sowinski, P. Fleming, T.R. Kershaw, J.D. Sinden, B.S. Meldrum, J.A. Gray, Contrasting effects of fetal CA1 and CA3 hippocampal grafts on deficits in spatial learning and working memory induced by global cerebral ischemia in rats, Neuroscience 72 (1996) 959–988.
[6] C. Iadecola, in: W.W. Totowa (Ed.), Mechanisms of Cerebral Ischemic Damage in Cerebral Ischemia: Molecular and Cellular Pathophysiology, Humana Press, NJ, 1999, pp. 3–32.

[7] Q. Jiang, Z.G. Zhang, G.L. Ding, L. Zhang, J.R. Ewing, L. Wang, R. Zhang, L. Li, M. Lu, H. Meng, A.S. Arbab, J. Hu, Q.J. Li, D.S. Pourabdollah-Nejad, A. Athiraman, M. Chopp, Investigation of neural progenitor cell induced angiogenesis after embolic stroke in rat using MRI, Neuroimage 28 (2005) 698–707.
[8] Q. Jiang, Z.G. Zhang, G.L. Ding, B. Silver, L. Zhang, H. Meng, M.D. Lu, S. Pourabdollah-Nejed, L. Wang, S. Savant-Bhonsale, L. Li, H. Bagher-Ebadian, J. Hu, A.S. Arbab, MRI detects white matter reorganization after neural progenitor cell treatment of stroke, Neuroimage 32 (2006) 90–1080.
[9] K. Jin, Y.J. Sun, L. Xie, X.O. Mao, J. Childs, A. Peel, A. Logvinova, S. Banwait, D.A. Greenberg, Comparison of ischemia-directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat, Neurobiol. Dis 18 (2005) 366–374.
[10] K.M. Kim, K. Park, S.W. Jeong, H.K. Park, K.H. Jung, S.T. Lee, L. Kang, Lee, D.K. Park, S.U. Kim, J.K. Roh, Human neural stem cells improved sensorimotor deficits in the adult rat brain with experimental focal ischemia, Brain Res. 1016 (2004) 145–153.
[11] C.A. Lepore, A. Bakshi, S.A. Swanger, M.S. Rao, I. Fische, Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord, Brain Res. 1045 (2005) 206–216.
[12] D. Lerouet, V. Berthat, B. Palmier, M. Plotkine, I. Margaill, Changes in oxidative stress, iNOS activity and neuropil infiltration in severe transient focal cerebral ischemia in rats, Brain Res. 958 (2002) 166–175.
[13] E.Z. Longa, P.R. Weinstein, S. Carlson, R. Cummins, Reversible middle cerebral artery occlusion without craniectomy in rats, Stroke 20 (1989) 84–91.
[14] J.D. Macklis, Transplanted neocortical neurons migrate selectively into regions of neuronal degeneration produced by chromophore targeted laser photolysis, J. Neurosci. 13 (1993) 3848–3863.
[15] M. Modo, R.P. Stroemer, E. Tang, S. Patel, H. Hodges, Effects of implantation site of stem cell grafts on behavioral recovery from stroke damage, Stroke 33 (2002) 2270–2278.
[16] M. Modo, P. Rezaei, P. Heuschling, S. Patel, D.K. Male, H. Hodges, Transplantation of neural stem cells in a rat model of stroke: assessment of short-term graft survival and acute host immunological response, Brain Res. 958 (2002) 70–82.
[17] B.A. Reynolds, S. Weiss, Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system, Science 255 (1992) 1707–1710.
[18] J.D. Sinden, F. Rashid-Doubell, T.R. Kershaw, A. Nelson, A. Chadwick, P.S. Jat, M.D. Noble, H. Hodges, J.A. Gray, Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischemia-lesioned hippocampus, Neuroscience 81 (1997) 599–608.
[19] J. Takahashi, T.T. Palmer, F.H. Gage, Retinoic acid and neurotropins collaborate to regulate neurogenesis in adult-derived neural stem cell culture, J. Neurobiol. 38 (1999) 65–81.
[20] H. Toda, J. Takahashi, A. Mizoguchi, K. Koyano, N. Hashimoto, Neurons generated from adult rat hippocampal stem cells from functional glutamatergic and GABAergic synapses in vitro, Exp. Neurol. 165 (2000) 66–76.
[21] T. Veizovic, J.S. Beech, R.P. Stroemer, W.P. Watson, H. Hodges, Resolution of stroke deficits following contralateral grafts of conditionally immortal neuroepithelial stem cells, Stroke 32 (2001) 1012–1019.