Intralesional mesenchymal stromal cell transplant in a rodent model of cortical cryoinjury

Ranjith K. Moorthy, Gift Ajay Sam, Sam Vijay Kumar[1], Geeta Chacko, Vikram Mathews[1], Ari G. Chacko, Alok Srivastava[1], Vedantam Rajshekhar

Departments of Neurological Sciences and [1]Haematology, Christian Medical College, Vellore, Tamilnadu, India

Abstract

Background: The effect of intralesional mesenchymal stromal cell (MSC) transplant in the subacute phase of brain injury has not been studied. **Aim:** To evaluate the role of intralesional transplant of mouse MSC following coldinduced cerebral cortical injury in mouse in improving neurological function. **Material and Methods:** Twelve mice (Swiss albino strain) received an intralesional injection of 2×10^6 mouse MSCs labelled with Bromodeoxyuridine (BrdU) and suspended in phosphate-buffered saline (PBS), 72 h after cerebral cryoinjury. Six mice received intralesional injection of PBS and served as controls. Neurological severity score (NSS) and rotarod treadmill test were used to perform serial assessments. **Results:** The mean NSS in the control group (n=5) on the first posttrauma day was 9.3±1.2 and it improved to 14.2±1.3 on day 28. The mean NSS in the MSC group (n=11) was 10.7±1.8 on the first posttrauma day and it improved to 16±1.1 on day 28 posttransplant. This difference was not found to be statistically significant when subgroup analysis of animals, where the assessments were blinded, was performed. There was no significant difference in the rotarod treadmill scores between the control and the MSC group at any time point. Few BrdU-positive cells could be identified in the periphery of the contusion up to day 10 posttransplant. **Conclusions:** Transplanted MSCs were shown to survive for at least 10 days after intralesional transplant in the cryoinjury model of the mouse cerebral cortex but the functional recovery observed in the experimental group was not statistically different from the controls.

Key words: *Brain injury, mesenchymal stromal cell, stem cell, transplant*

Address for correspondence:
Dr. Ranjith K Moorthy,
Department of Neurological Sciences,
Christian Medical College,
Vellore-632 004, India.
E-mail: ranjith@cmcvellore.ac.in

Received : 24-10-2010
Review completed : 26-11-2010
Accepted : 12-01-2011

Introduction

With the improvement in management paradigms, there has been a definite decline in the mortality rates in patients with traumatic brain injury (TBI). However, a significant number of survivors are disabled due to the neurological deficits.[1] Since the first report demonstrating the role of mesenchymal stem cell (MSC) transplant in a model of brain injury,[2] there has been considerable progress in this area with several reports of short- and long-term functional improvements after transplant of adult stem cells in a controlled cortical impact of rodent brain injury.[3-18] Bone marrow stromal cell transplant has also been shown to result in the improvement of neurologic function after a freezing injury to the rat cerebral cortex.[19,20] Most of the studies report transplant of cells at 24 h after creation of injury. We attempted to evaluate neurological functional recovery and cell survival in a cold injury model in the

Access this article online	
Quick Response Code:	Website: www.neurologyindia.com
	DOI: 10.4103/0028-3886.84340

cerebral cortex of mice after intralesional transplant of mouse MSCs at 72 h post injury.

Material and Methods

Twenty eight adult female Swiss albino mice weighing 20–35 g were used for the experiment. The animals were housed in the Central Animal Facility of the institution and were maintained in an optimal environment with 24 h dark–light cycles. The surgical and transplantation protocols were approved by the animal ethics committee as well as the institutional review board.

Creation of cold injury

Under general anesthesia (Inj. ketamine 100 mg/kg and Inj. xylazine 10 mg/kg), the mice were immobilized on a stereotactic frame (TMB systems, Germany) after ensuring that the toe pinch withdrawal reflex was lost. The respiratory rate and color of the extremities was observed during the procedure. A midline incision was made over the scalp to expose the skull from the coronal suture to the lambdoid suture. Using an electric dental drill, a 5 mm craniotomy was performed just right of midline and in between the coronal suture and lambdoid suture to expose the dura mater. A hollow copper cylinder with a 3 mm tip was cooled to −50°C to −55°C using a mixture of dry ice and acetone. This precooled hollow copper cylinder was placed over the intact dura and contact maintained for 3 min. This model of cryoinjury was a modification of previously reported protocols.[21,22] The wound was sutured and the animals were returned to their cages.

Preparation of cell suspensions

Five male mice were sacrificed and immediately afterwards, the femur and tibia were removed. The femur and tibia were crushed in a sterile mortar and suspended in a medium consisting of Iscove's modified Dulbecco's medium (IMDM), containing 20% foetal bovine serum (FBS) with 2 g/L. HEPES buffer, hydrocortisone and 2-mercaptoethanol. Penicillin and streptomycin were added to the medium. The mononuclear fraction was then separated using Ficoll separation protocol and the interphase cells seeded into a T75 flask at 15×10^6 cells/cm^2. Adherent cells were passaged at 80%–90% confluence. After the second passage, the cells were confirmed to be MSCs by immunophenotyping and thereafter used for transplantation procedures before the third passage. For immunophenotyping, the cells were washed with phosphate-buffered saline (PBS) and were incubated with a panel of antibodies to Sca-1, CD49, CD73, CD29, and CD44 (BD Pharmingen, San Jose, CA, USA) and CD105 (Southern Biotech, Birmingham,AL, USA) conjugated with fluorescein isothiocyanate(FITC) or phycoerythrin (PE). After 20 min of incubation, the cells were washed and analyzed using FACSCalibur (Becton Dickenson, Mansfield, MA, USA). A total of 10,000 events were acquired for each analysis. Data analysis was performed using CellQuestPro software (San Jose, CA, USA).

Three days before the transplant, bromodeoxyuridine (BrdU) was added at a 30 mM concentration to the MSC cultures. On the day of the transplant, the cells were trypsinized, assessed for viability, and counted. The cells were resuspended in PBS at a concentration of 1×10^6 cells/10 µL for transplant procedure.

Transplant

Seventy-two hours postinjury, the animals were re-anesthetized using the same protocol described above and immobilized in the stereotactic frame. The wound was reopened and the craniotomy exposed. Twelve animals in the experimental group received 2×10^6 MSCs with a total of 20 µL of suspension being injected using a 10 µL Hamilton microsyringe (Hamilton Inc., NV, USA); at the anterior and posterior pole of the craniotomy, the tip of the needle at a depth of 1 mm from the dural surface. Six animals in the control group received PBS in the same volume using the same procedure. The wound was re-sutured and the animals were returned to their cages.

Clinical monitoring

The animals were monitored on day 1 and 3 postinjury and on days 7, 14, 21, and 28 after the transplant.

The neurological severity score (NSS) consisted of 6 subdivisions: spontaneous activity, asymmetry of limb movements, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch.[23] Each subdivision was scored from 0 to 3 and the total score calculated. The higher the score better was the performance of the animal. The NSS assessment was performed by an observer who was blinded to the previous score of the animal in the first half of the experiment and the observer was blinded to the treatment received by the animal in the second half. The NSS recovery rate was computed as {(posttransplant NSS − pretransplant NSS)/ pretransplant NSS}×100.

The animals were subjected to a paradigm consisting of uniform acceleration at 4 rounds per min using the rotarod treadmill. They were pretrained for 3 days before the injury was induced to obtain their baseline scores, and serial measurements were performed thereafter. The raw scores (in seconds) obtained on the rotarod treadmill were converted to percentage of the baseline scores to analyze the data.

Pathology

At 28 days after the transplant, the mice were sacrificed and the heart perfused with 4% formaldehyde solution. In addition, two animals each at 3, 7, and 14 days posttransplant were sacrificed for histologic studies. The brains were then removed. Gross and microscopic examination was performed. Sections of the brain through the area of contusion and at its periphery were obtained and examined using hematoxylin and eosin staining and avidin–biotin–peroxidase immunohistochemistry for BrdU to detect the presence of the transplanted cells. The primary antibody used was monoclonal anti-BrdU (DAKO, 1:200, DAKO Patts, Denmark) and the secondary antibody was biotinylated anti-rabbit mouse antibody (1:200, DAKO Patts, Denmark).

Statistical analysis

The mean and standard deviation for the NSS, NSS recovery rate as well as rotarod scores were calculated for each group at each time point. Independent samples *t* test and Mann–Whitney *U* test was used to test significance. *P* value of <0.01(in order to minimize the alpha error) was considered significant for comparison of mean scores across each time point.

Results

Neurological severity score

All the animals showed deterioration in the NSS in the postinjury period. The mean score in the control group (n=5) on posttrauma day 1 was 9.3±1.2 and it improved to 14.2±1.3 on day 28 following injection of PBS. The mean score in the MSC transplant group (n=11) was 10.7±1.8 on posttrauma day 1 and it improved to 16±1.1 on day 28 after the injection of the MSCs, the difference being statistically significant compared with the controls (*P*=0.007). The score on day 21 posttransplant also showed a statistically significant difference between the controls and MSC transplant group (*P*=0.006). However, on a subgroup analysis of the mice in whom the assessments were completely blinded (n=6), there was no statistically significant difference between the controls and MSC transplant group at any time point, although the NSS of the MSC transplant group tended to be higher than the control group from day 14 posttransplant [Table 1].

Rotarod test

All the animals showed deterioration in the rotarod scores on day 1 postinjury that improved, albeit partially, by 72 h, the time at which they received the transplant. There was no significant difference in the rotarod treadmill scores between the control and the MSC transplant group at any time point, although the latter group tended to perform better than the control group from day 14 posttransplant. The results are summarized in Table 2.

Mortality

Four animals died immediately following induction of the cold injury. One animal each in the experimental and control group died within the first week after the transplant procedure.

Pathological examination

Gross and microscopic examination of all the animals showed a depressed area on the cortical surface corresponding to the site of contusion. There was microscopic evidence of cortical injury with cavitation and formation of a glial scar on hematoxylin and eosin staining [Figure 1]. Immunohistochemistry for BrdU revealed the presence of positively stained nuclei at the site of the contusion on day 3 and 10 [Figure 2] posttransplant, while there was no evidence of BrdU labeling on the sections on day 28. As early as day 7 posttransplant, staining of nuclei within the choroid plexus as well as the capillary walls was observed on BrdU immunostaining [Figure 3].

Discussion

This study shows that following intralesional transplant of MSCs in the subacute phase onday 3 after cryoinjury, the cells survive up to 10 days posttransplant. The functional improvement seen in the MSC group seems to occur from day 14 onward,

Table 1: Neurological severity score in the control and experimental groups

	Control (n=5)	Mesenchymal stromal cell group (n=11)	*P* value
Posttrauma day 1	9.3±1.2	10.7±1.8	0.12
Posttrauma day 3	9.5±3.3	11.2±2.3	0.28
Posttransplant day 7	12±1.8	12.3±1.7	0.67
Posttransplant day 14	13.5±1	13.7±1.7	0.8
Posttransplant day 21	13.1±1	14.8±1.1	0.007*
Posttransplant day 28	14.2±1.3	16±1.1	0.007*

*On subgroup analysis of the treated animals when the observer was blinded, there was no statistically significant difference between controls and mesenchymal stromal cell group on posttransplant day 21 and 28.

Table 2: Rotarod scores of the control and experimental group

	Control (n=5)	Mesenchymal stromal cell group (n=11)	*P* value
Baseline	100	100	
Posttrauma day 1	51.4±41.3	43.5±37.3	0.46
Posttrauma day 3	57.9±58.6	58.4±41.6	0.95
Posttransplant day 7	82.1±24.6	72±34.8	0.61
Posttransplant day 14	70.2±36	80.6±24.9	0.33
Posttransplant day 21	76.4±12.9	86±23.4	0.36
Posttransplant day 28	81.1±19	91.1±16.4	0.33

although it was not statistically different from that observed in the controls.

Conflicting results of mesenchymal stem cell transplant in brain injury

Several cell types from embryonic, fetal, as well as adult sources have been evaluated in preclinical studies in rodent models of brain injury.[1,2,22] Adult stem cells, such as MSCs, have the advantage of not requiring immunosuppression to prevent graft rejection.[1,18] In a series of publications Chopp and co-workers reported that intralesional, intra-arterial, or intravenous transplant of rat or human-derived MSCs at 24 h following cortical impact injury results in improvement of neurologic function in rats that is sustained up to 6 months.[4-6,8-15] After the initial reports, the same investigators reported that the use of MSCs in combination with statins could have a synergistic effect by increasing the quanta of MSCs delivered to the injury site and promoting angiogenesis.[16,17] This group has also demonstrated reduction in lesion volume and improved functional outcomes by using collagen scaffolds populated with MSCs.[7] In the only report on MSC transplant in mice after controlled cortical impact injury, intravenous injection of MSCs resulted in improved neurological function.[18]

In contrast to these findings, Harting *et al*[24] reported that only about 0.0005% of MSCs infused intravenously reached the cerebral parenchyma and almost no donor cells could be identified in the brain tissue 2 weeks postinfusion. They also did not report any functional improvement after intravenous infusion of MSCs following cortical impact injury. Thus there is a role for evaluating nonsystemic routes of delivery of MSC to bypass the "filter effect" of the pulmonary circulation.

Intralesional MSC delivery 1 h following focal freezing injury in rats, has been shown to improve

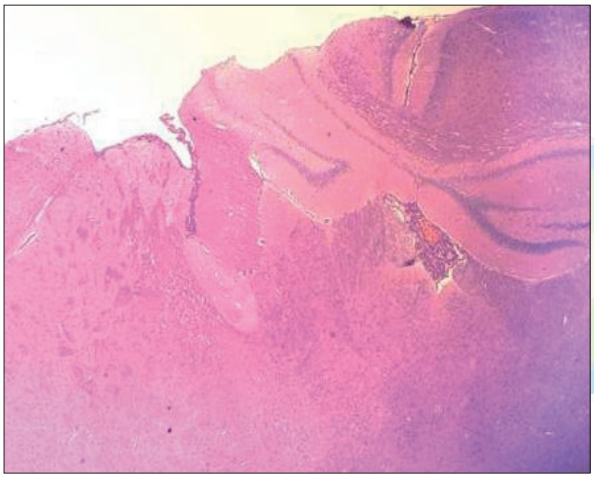

Figure 1: Photomicrograph of a hematoxylin and eosin stained section of a mouse brain at day 28 following injury showing evidence of a cavitation at the site of the injury with underlying scar. Note the deformation of the hippocampus on the involved side

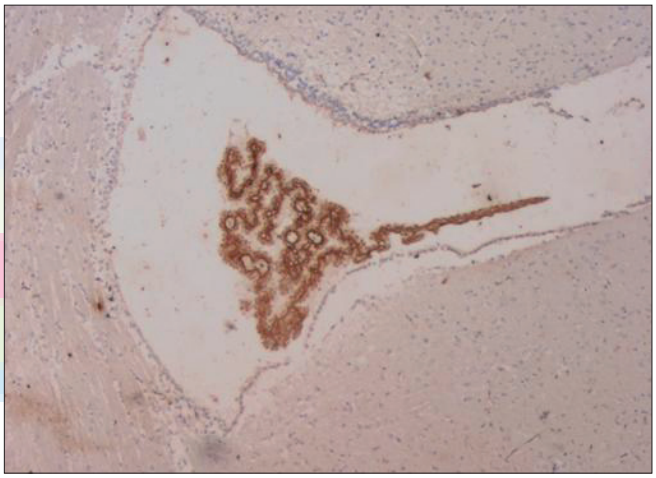

Figure 3: Photomicrograph of BrdU immunostained section of a mouse brain at day 7 post transplant showing dense labelling of the nuclei within choroid plexus epithelium

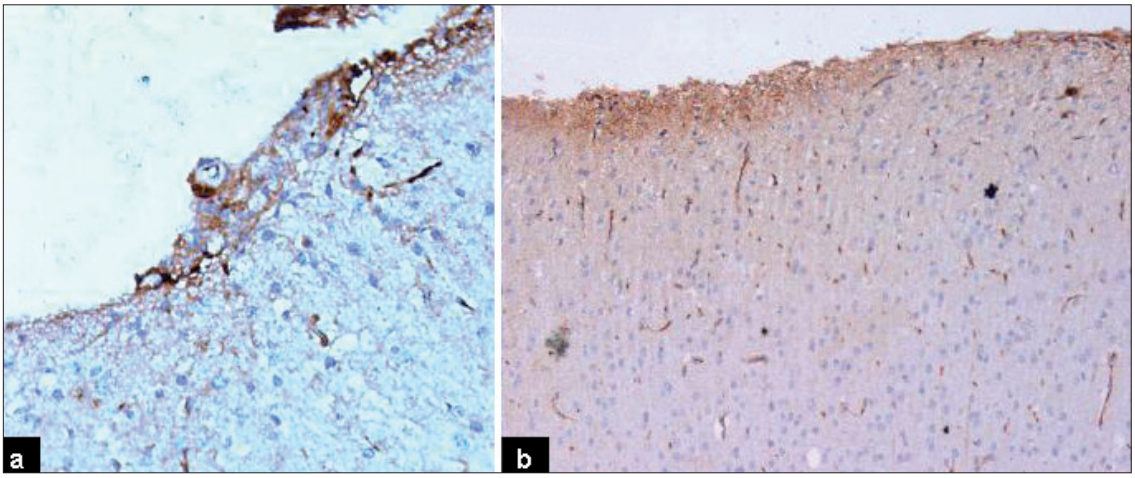

Figure 2: Photomicrographs of BrdU immunostained sections showing (a) Presence of positively stained nuclei of cells on the surface and just below the cortex on day 7 following transplant of the MSCs (40x) and (b) Presence of positively labelled cells in the subcortical region on day 10 following transplant of MSCs (20x). Note the presence of positively labelled cells in the capillary walls as well

metabolic parameters that correlated with functional improvement.[20] Enhancement of survival of transplanted MSCs in the central nervous system has been demonstrated by use of salvianolic acid B, basic fibroblast growth factor and by addition of neurotrophic factors into the culture medium.[3,10,25] It remains to be seen whether neurally modifying the MSC pretransplantation would enhance survival.[6,26] In our study, the BrdU-labelled MSCs could be demonstrated only for a short period of up to 10 days after the time of transplant, and the improvement in function seen in the transplanted group was not statistically different from that seen in the control group. It is possible that the number of animals in each group was small and with a larger sample size, a statistically significant difference between the 2 groups could be detected.

Possible causes for discrepancy in results

Most studies have reported early transplant of MSCs within 1–24 h of the injury.[4-14,20] There is a single report of MSC transplant at 7 days after injury.[15] Transplant immediately after or at 24 h after injury may result in the cells having to survive the inflammatory response mounted in response to the injury and this may be more detrimental than beneficial to cell survival.

The timing of 72 h was chosen in our study as this is the time the inflammatory and cellular response to injury begins to stabilize, providing the ideal niche for survival of the transplanted cells. The transplant at 72 h after injury also aids in the evaluation of spontaneous recovery of neurological function that may occur within the first 3 days of the injury. Indeed, this was observed in all our animals both in the NSS and rotarod assessment, indicating that this would be an important confounder to consider while reporting or reviewing literature on the beneficial effects of the stem cell transplantation. The assessment and reporting of the NSS recovery rate that takes into account the baseline score of each animal and the amount of recovery that actually occurs has not been included in previous studies. In our study, although the raw NSS score seemed to show that the MSC transplant group performed better than the controls, the recovery rate was in fact higher among the control group animals, although statistically not significant.

A review of previously reported data of rotarod scores on MSC transplant indicates that the control animals also showed recovery of function but the recovery of neurological function in the transplanted group in rotarod treadmill test was better than in the control group.[6,9,11,13,14,16] Our findings are similar to those of Harting *et al*,[24] who also did not find any significant improvement in rotarod test performance. In another study on MSC transplant in cortical impact injury in mice, there was statistically significant improvement in the NSS in the experimental group compared with the controls by day 21, but this effect was lost by day 35.[18] However, improved performance in spatial learning tasks, such as water maze test, was noted in the transplanted group at more than 1 month after the transplant.[18] The findings on the water maze test are in contrast to the improved rotarod scores reported sooner after the transplant.[6,9,11-14,16] Thus interpretation of the observed functional outcome following MSC transplant in an experimental model could depend on the type of assessment performed and the interval from the injury at which function is assessed.

Fate of transplanted cells

Progressive decline in the number of transplanted MSCs detected by immunolabeling over the first 3 weeks following transplant has been reported following a freezing injury to the rat cortex.[20] It has been reported that MSCs transplanted into the normal adult rat brain are rejected by an inflammatory response and subsequently their markers are transferred to the recipient cells.[27] To track the fate of the transplanted cells several markers have been used. In our study, BrdU was used to label the cells but nuclear staining of BrdU was not demonstrated at 28 days posttransplant. Although this would lead to the conclusion that the cells do not survive, it has been reported that transplanted cells do transfer their BrdU to the recipient cells (as we have demonstrated in the choroid plexus of the recipient animals) in the intact brain and it may not be the most efficient method to monitor transplanted cells. An inflammatory response mounted against the transplanted cells probably results in their destruction and transfer of the label into host cells.[27] Infrared macroscopic cell tracking, GFP labelling of cells, or the use of MRI to track transplanted cells labelled with iron particles have been reported as alternative methods to track the fate of transplanted cells.[24,27] Future studies may need to incorporate more than one of these strategies to monitor the fate of the transplanted cells.

Acknowledgment

This study was supported by a grant from the Department of Biotechnology, Government of India.

References

1. Longhi L, Zanier ER, Royo N, Stochetti N, McIntosh TK. Stem cell transplantation as a therapeutic strategy for traumatic brain injury. Transpl Immunol 2005;15:143-8.
2. Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop D. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats, similarities to astrocyte grafts. Proc Natl Acad Sci USA 1998;95:3908-13.
3. Bhang SH, Lee YE, Cho SW, Shim JW, Lee SH, Choi CY, *et al*. Basic fibroblast growth factor promotes bone marrow stromal cell transplantation-mediated neural regeneration in traumatic brain injury

Biochem Biophys Res Commun 2007;359:40-5.
4. Lu D, Mahmood A, Wang L, Li Y, Lu M, Chopp M. Adult bone marrow stromal cells administered intravenously to rats after traumatic brain injury migrate into brain and improve neurological outcome. Neuroreport 2001;12:559-63.
5. Lu D, Li Y, Wang L, Chen J, Mahmood A, Chopp M. Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J Neurotrauma 2001;18:813-9.
6. Lu D, Mahmood A, Wang L, Rafiq T, Chopp M. Neural and marrow-derived stromal cell sphere transplantation in a rat model of traumatic brain injury J Neurosurg 2002;97:935-40.
7. Lu D, Mahmood A, Qu C, Hong X, Kaplan D, Chopp M. Collagen scaffolds populated with human marrow stromal cells reduce lesion volume and improve functional outcome after traumatic brain injury. Neurosurgery 2007;61:596-602.
8. Mahmood A, Lu D, Wang L, Li Y, Lu M, Chopp M. Treatment of traumatic brain injury in female rats with intravenous administration of bone marrow stromal cells. Neurosurgery 2001;49:1196-1203.
9. Mahmood A, Lu D, Yi L, Chen JL, Chopp M. Intracranial bone marrow transplantation after traumatic brain injury improving functional outcome in adult rats. J Neurosurg 2001;94:589-95.
10. Mahmood A, Lu D, Wang L, Chopp M. Intracerebral transplantation of marrow stromal cells cultured with neurotrophic factors promotes functional recovery in adult rats subjected to traumatic brain injury. J Neurotrauma 2002;19:1609-17.
11. Mahmood A, Lu D, Lu M, Chopp M. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. Neurosurgery 2003;53:697-702.
12. Mahmood A, Lu D, Chopp M. Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. J Neurotrauma 2004;21:33-9.
13. Mahmood A, Lu D, Chopp M. Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. Neurosurgery 2004;55:1185-93.
14. Mahmood A, Lu D, Qu C, Goussev A, Chopp M. Human marrow stromal cell treatment provides long-lasting benefit after traumatic brain injury in rats. Neurosurgery 2005;57:1026-31.
15. Mahmood A, Lu D, Qu C, Goussev A, Chopp M. Long-term recovery after bone marrow stromal cell treatment of traumatic brain injury in rats. J Neurosurg 2006;104:272-7.
16. Mahmood A, Lu D, Qu C, Goussev A, Chopp M. Treatment of traumatic brain injury with a combination therapy of marrow stromal cells and atorvastatin in rats. Neurosurgery 2007;60:546-53.
17. Mahmood A, Goussev A, Lu D, Qu C, Xiong Y, Kazmi H, *et al*. Long-lasting benefits after traumatic brain injury (TBI) in rats with combination therapy of marrow stromal cells (MSCs) and simvastatin. J Neurotrauma 2008;25:1-8.
18. Qu C, Mahmood A, Lu D, Goussev A, Xiong Y, Chopp M. Treatment of traumatic brain injury in mice with marrow stromal cells. Brain Res 2008;1208:234-9.
19. Cho YH, Kim HS, Lee KH, Lee YE, Chang JW. The behavioural effect of human mesenchymal stem cell transplantation in cold brain injured rats. Acta Neurochir Suppl 2006;99:125-32.
20. Mori K, Iwata J, Miyazaki M, Nakao Y, Maeda M. Functional recovery of neuronal activity in rat whisker-barrel cortex sensory pathway from freezing injury after transplantation of adult bone marrow stromal cells. J Cereb Blood Flow Metab 2005;25:887-98.
21. Hortobagyi T, Hortobagyi S, Gorlach C, Harkany T, Benyo Z, Gorogh T, *et al*. A novel brain trauma model in the mouse: Effects of dexamethasone treatment. Eur J Physiol 2000;441:409-15.
22. Lacza Z, Horvath E, Busija DW. Neural stem cell transplantation in cold lesion: A novel approach for the investigation of brain trauma and repair. Brain Res Protocols 2003;11:145-54.
23. Garcia JH, Wagner S, Kiu KH, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Stroke 1995;26:627-35.
24. Harting MT, Jimenex F, Xue H, Fischer UM, Baumgartner J, Dash PK, *et al*. Intravenous mesenchymal stem cell therapy for traumatic brain injury, J Neurosurg 2009;110:1189-97.
25. Bi XB, Deng YB, Gan DH, Wang YZ. Salvianolic acid B promotes survival of transplanted mesenchymal stem cells in spinal cord-injured rats. Acta Pharmacol Sin 2008;29:169-76.
26. Alexanian AR, Maiman DJ, Kurpad SN, Gennarelli TA. *In vitro* and *in vivo* characterization of neurally modified mesenchymal stem cells induced by epigenetic modifiers and neural stem cell environment. Stem Cells Dev 2008;17:1123-30.
27. Coyne TM, Marcus AJ, Woodbury D, Black IB. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 2006;24:2483-92.

How to cite this article: Moorthy RK, Sam GA, Kumar SV, Chacko G, Mathews V, Chacko AG, *et al*. Intralesional mesenchymal stromal cell transplant in a rodent model of cortical cryoinjury. Neurol India 2011;59:573-8.

Source of Support: Department of Biotechnology, Government of India, **Conflict of Interest:** None declared.