Clinical Neurology and Neurosurgery 115 (2013) 1003–1008

Contents lists available at SciVerse ScienceDirect

Clinical Neurology and Neurosurgery

journal homepage: www.elsevier.com/locate/clineuro

Stem cell therapy: A clinical trial of stroke

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ARTICLE INFO

Article history:
Received 2 July 2012
Received in revised form
18 September 2012
Accepted 20 October 2012
Available online 24 November 2012

Keywords:
Stroke
Stem cell transplantation
Regenerative medicine

ABSTRACT

Background: The alarming disability burden and a high prevalence rate of stroke in India has encouraged the researchers to develop regenerative therapies to reduce clinical deficits. This study evaluates safety, feasibility and efficacy of autologous mononuclear and mesenchymal cell transplantation in stroke patients evaluated on clinical scores and functional imaging (fMRI and DTI).
Methods: Forty ($n = 40$) stroke patients were recruited with the inclusion criteria as: 3 months to 2 years of index event, power of hand muscles of at least 2; Brunnstrom stage: 2–5; conscious and comprehendible. Fugl Meyer (FM), modified Barthel Index (mBI), Medical Research Council (MRC) grade for strength, Ashworth tone scale and functional imaging was used for assessments at baseline, 8 weeks and 24 weeks. 50–60 million cells in 250 ml saline were infused intravenously over 2–3 h.
Results: The safety test profile was normal with no mortality or cell related adverse reactions in stem cell patients. Among outcome parameters, only modified Barthel Index (mBI) showed statistical significant improvement ($p < 0.05$) in the stem cell group. An increased number of cluster activation in Brodmann areas BA 4, BA 6 was observed post stem cell infusion indicating neural plasticity.
Conclusion: Autologous intravenous stem cell therapy is safe and feasible. Stem cells act as "scaffolds" for neural transplantation and may aid in repair mechanisms in stroke.

1. Introduction

1.1. Stem cells in CNS regeneration and plasticity

Cells of the brain and nervous system were thought to be incapable of regeneration. However in the last decade, evidence of neurogenesis in the adult brain has been demonstrated [1]. Adult stem cells are multipotent cells found in developed organisms, which replace cells that have died or lost function with special mention to neurological diseases such as stroke [2,3]. Human cells that have been used in these studies fall into 3 categories, neural stem/progenitor cells (NPCs) cultured from fetal tissue, immortalized neural cell lines and hematopoietic/endothelial progenitor or stromal cells isolated from bone marrow, umbilical cord blood, peripheral blood, or adipose tissue [4]. The mechanisms by which the cell transplantation might improve stroke deficits are that transplanted cells may integrate into the host circuitary, reduce death of host cells, induce host brain plasticity, increase neovascularization and recruitment of endogenous progenitors [5,6]. The knowledge gained about brain plasticity following stroke or brain damage needs to be linked with neuroregenerative strategies such as stem cells thus promoting neurobiological recovery processes boosting repair at clinical and functional levels.

1.2. Hematopoietic/mononuclear stem cells

Hematopoietic stem cells include population of endothelial stem, progenitor and CD34+ cells. Studies have demonstrated increased angiogenesis in penumbral tissue following CD34+ cell transplantation, whether given systemically or by the intracerebral route [7]. These studies have also demonstrated a good functional recovery, reduced infarct size and homing in mechanism of stem cells in the peri-infarct zone. Some of the experimenters also used an antiangiogenic compound, endostatin, administered 7 days after CD34+ cell transplantation and demonstrated that endogenous neurogenesis was suppressed by diminishing angiogenesis, thus suggesting a possible role for CD34+ cells in angiogenesis-mediated neural plasticity post-stroke [8,9]. A

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http://dx.doi.org/10.1016/j.clineuro.2012.10.015

number of clinical trials are currently underway investigating the role of bone-marrow-derived stem cell therapy at different stages of ischemic stroke, utilizing different methods of delivery [10].

1.3. Mesenchymal stem cells

It is evident from preclinical studies that MSCs secrete insulin-like growth factor-1 followed by an enhanced expression of VEGF, EGF, and basic fibroblast growth factor within endogenous neural cells, which results in reduced infarct injury. The systemic injection of MSCs was associated with direct anti-apoptotic effects and modulation of inflammatory responses within the ischemic tissue resulting in reduced neural damage in the peri-infarct zone, where glial scar formation has been described to be reduced after MSC transplantation [11]. MSC therapy results in enhanced levels of endogenous growth factors such as VEGF and have been reported to stimulate angiogenesis along the ischemic boundary zone via mechanisms involving enhanced expression of both endogenous VEGF and VEGF receptors [12,13].

1.4. Stem cells for stroke

Several reviews, six published clinical trials and one case study have been reported with stem cell therapy in ischemic and hemorrhagic stroke [14,15]. Stem cell transplantation has emerged as 'hope' to cure functional deficits after stroke. It is postulated that stem cells operate not through a unidirectional mechanism (e.g., generating neurons) but rather as cellular mediators of a multitude of biological activities that could provide a favorable outcome for diverse nervous disorder. This present research studied safety and efficacy of intravenous autologous bone marrow derived mononuclear and culture expanded mesenchymal stem cells in stroke. We also studied the comparison between the two type of cells measured on clinical and functional imaging evaluation.

2. Methods

This was an unblinded non randomized case control study in which we included patients with diagnosed stroke from 3 months to 2 years of index event, Brunnstrom stage of recovery between 2 and 5, age: 18–65 years, NIHSS (National Institute of health stroke scale) of between 4 and 15, conscious and able to follow commands. We excluded the following diseases: autoimmune disorders, immune-compromised states, hematological disorders, chronic liver and renal failure, progressive neurological worsening, neoplasia, contraindication to MRI and pregnancy. The patients were examined by neurologist and neurophysiotherapist for strength, tone (modified Ashworth), Fugl Meyer (FM) scale for upper limb, Edinburgh handedness inventory, modified Barthel Index (mBI) [16,17] and functional MRI scanning was performed at baseline, 8 and 24 weeks of stem cell transplantation.

The research was cleared by Institute Review Board (IRB). Prior to stem cell therapy, patients were screened and educated about stem cells and bone marrow aspiration technique. Written informed consent was obtained, complete medical history, examination and baseline laboratory tests were performed. We recruited forty ($n = 40$) stroke patients with the above inclusion criteria. Twenty patients ($n = 20$) were given stem cells followed by 8 weeks of physiotherapy, serving as experimental group and twenty patients ($n = 20$) were administered with physiotherapy regime alone (Fig. 1).

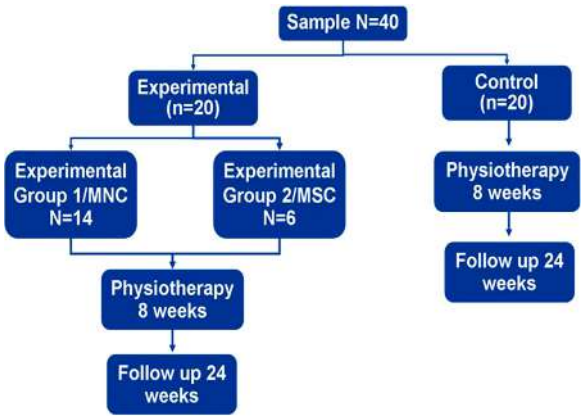

Fig. 1. Flow chart showing study methodology.

2.1. Procedure

2.1.1. Bone marrow aspiration, separation and transplantation of mononuclear stem cells (MNC)

Bone marrow (approx 40–50 ml) was aspirated under aseptic conditions from the posterior superior iliac crest in fourteen ($n = 14$) stroke patients. Bone marrow aspirate was diluted with phosphate buffered saline, layered over ficoll density medium and centrifuged at 1800 rpm for 25 min. Bone marrow mononuclear cells (BM-MNC) layer was collected, their sterility and viability was maintained before transplantation. MNC count and number of CD34+ cells (flow cytometry method) were counted for each patient. The whole procedure took approximately 2–3 h [18].

2.1.2. Bone marrow aspiration, expansion and transplantation of mesenchymal stem cells (MSC)

We transplanted six stroke ($n = 6$) patients out of twenty with culture expanded mesenchymal stem cells. The process of mesenchymal stem cells expansion started with the collection of mononuclear stem cell layer from the bone marrow aspirate as described above. The collected mononuclear cell layer was plated at a density of 1×10^6 cells/cm^2 with Stem Pro MSC SFM Basal Medium (A-10334, Stem Pro Medium by Invitrogen) [19] in a T-25 tissue culture flask and incubated at 37 °C/5% CO_2. The cells were harvested and seeded at 3000 cells/cm^2 or 10,000 cells/cm^2 and harvested by using TrypLE^{TM} Express (Invitrogen) at confluency. The cells were counted using flow cytometry and reseeded at the same seeding density till the final dose of cells were achieved. The non adherent cells were removed after 24 hrs and fresh media was changed every 3 days till confluency. The cells were trypsinized and sub cultured. All samples were tested for mycoplasma and endotoxins during the expansion. The whole procedure took around 23 ± 3 days for the cell expansion.

2.1.3. Cell infusion

An aseptic technique of infusion was followed in which the cells were given in a sterile 50 ml syringe which was directly dissolved in 250 ml of saline and infused intravenously in the medial cubital vein. The infusion lasted for 3 h. Around 50–60 million cells were infused which is similar to the ongoing clinical trials of stroke [8,13]. Patients were evaluated for safety i.e., laboratory tests (Hb%, RBC, WBC, platelets, liver and kidney function tests, PT) at 1st, 3rd, 7th day and 24 weeks of transplantation.

2.1.4. Physiotherapy regime

The physiotherapy regime was administered to all forty patients ($n = 40$), which was based on the motor imagery incorporating

Table 1
Demographics and baseline characteristics between experimental and control groups.

		Experimental	Control
Number		20	20
Sex ratio	Male	18	17
	Female	02	03
Type	Ischemia	18	19
	Hemorrhage	02	01
Side	Right	14	12
	Left	06	08
Sub type	Cortical	15	13
	Subcortical	05	07
Stroke classification	Large artery	09	11
	Small vessel	05	04
	Cardioembolic	–	02
	Stroke of other determined etiology	03	01
	Stroke of undetermined etiology	03	02
Age		45.1 ± 12.1	45.15 ± 11.8
Time of stroke		9.6 ± 3.2	8.5 ± 3.3
Volume		20.1 ± 13.4	20.1 ± 15.1
MRC		2 ± 0.59	2 ± 0.63
Ashworth		2 ± 0.68	2 ± 0.67
FM		18.6 ± 7.4	18.9 ± 7.6
mBI		46.1 ± 10.1	46.9 ± 10.1
FA ratio		0.49 ± 0.16	0.47 ± 0.17
FN ratio		0.29 ± 0.18	0.27 ± 0.15
FL ratio		0.23 ± 0.21	0.22 ± 0.23
% Signal intensity		1.18 ± 0.41	1.10 ± 0.61

learning and repetition principle of motor learning [20]. The intervention period was for 5 days/week for eight weeks for 60–90 min.

2.2. Functional MRI acquisition

MRI scans were done on 1.5 T MR scanner (Avanto, M/s. Siemens Medical Solutions, Germany) using gradient echo planar imaging (EPI) sequence with a total of 90 whole brain EPI measurements (TR = 4520 ms, TE = 44 ms, slices = 31, slice thickness = 4 mm). Multiplanar 3D sequence of 176 contiguous slice thickness = 1.0 mm were also acquired. Block design with alternate baseline and activation cycles was used in which the subjects performed motor task with paretic hand followed by bilateral hand, with self paced (minimum 0.5 Hz) fist clenching/extension of the wrist/extension of the MCP joints of the hands. This protocol has been widely discussed elsewhere [21].

3. Results

3.1. Establishment of safety

The mean cell viability of both the type of cells at transplantation was 98% which was performed with Trypan blue stain and the cells were sterile and endotoxin free during expansion and at transplantation. The routine laboratory tests (Hb%, TLC, DLC, platelets, prothrombin time, liver and kidney function tests) at 1st, 3rd and 7th day of the stem cell transplantation were within normal limits for all patients. There were no early or late adverse reactions during and after transplantation. The mean CD34+ count of MNC was 0.28% with mean 55.4×10^6 million cells where as mesenchymal cells expressed CD 90, CD 73, CD 105 and were negative for class HLA II. The mean CD 90, CD 73 and CD 105 were 61%, 57.1% and 40% respectively.

3.2. Clinical results

In the stem cell group (male:female = 15:5), (mean age = 45.05 ± 12.1), the mean baseline FM score was 18.6 ± 7.45, at 8 weeks was 30.9 ± 9.7 and at 24 weeks was 38.2 ± 9.77. We observed a statistical significant improvement in FM and mBI between baseline and 8 weeks, 8 and 24 weeks ($p < 0.05$) (Table 1). The strength and Ashworth tone scale did not show significant improvement between baseline and 8 weeks and 8–24 weeks. Patients showed statistically significant improvement when transplanted with stem cells ($p = 0.001$, $t = -18.157$). The male to female ratio in control group was 17:3 with mean age = 45.45 ± 9.7. The mean FM score was 18.9 ± 7.60, 29.45 ± 9.1 and 35.65 ± 8.57 at baseline, 8 and 24 weeks respectively. The mean mBI was 46.95 ± 10.04, 58.4 ± 9.3 and 68.4 ± 9.2 at baseline, 8 and at 24 weeks respectively. These patients also showed statistically significant improvement between (baseline and 8 weeks), (8 and 24 weeks) weeks ($p < 0.05$) for both FM and mBI scores. Ashworth tone scale and MRC for hand muscles remained statistically insignificant.

Laterality index of the ipsilesional BA 4 and BA 6, % signal intensity change, hemodynamic response in the lesioned cortex from the BOLD activation scans and fractional anisotropy (FA) ratio, fiber number (FN) ratio and fiber length (FL) ratio in each ROI of both the hemispheres were calculated from tensor imaging. Statistical significant difference was observed in the laterality index (LI) of ipsilesional BA 4 and 6, between baseline to 8 weeks ($p = 0.004$), between baseline and 24 weeks ($p = 0.001$) in both the groups.

3.3. Comparison between stem cell and control group

The baseline clinical and radiological scores between the experimental and control group were statistically insignificant (2 sample *t*-test) which suggested that the two groups can be compared to study the effectiveness of the therapy at 8 and 24 weeks. There was no significant difference in FM, Ashworth, MRC between the two groups at 8 and 24 weeks. Modified Barthel Index was statistically significant at 24 weeks ($p = 0.05$) only (Table 2). When the experimental group 1 was compared with control subjects, it was observed that modified Barthel Index was statistically significant only at follow up (24 weeks) and the other outcome measures were insignificant. The LI index of BA 4 and BA 6 between stem cell and control groups were also statistically insignificant at 8 weeks and 24 weeks ($p = 0.99$, $p = 0.78$ respectively). There was no statistically significant ($p > 0.05$) change in the FA ratio, fiber length (FL) ratio and fiber number (FN) ratio between the two groups at 8

Table 2
Mean scores and analysis of clinical scores at 8 and 24 weeks.

	Experimental	Control	Exp vs. control (p value)	Exp 1	Exp 2	Exp 1 vs. control (p value)	Exp 2 vs. control (p value)	Exp 1 vs. exp 2 (p value)
8 weeks								
FM	30.9 ± 9.7	29.45 ± 9.1	0.63	31.7 ± 10.7	29.0 ± 7.4	0.52	0.90	0.31
mBI	63.9 ± 13.2	58.4 ± 9.3	0.43	65.75 ± 13.2	59.5 ± 11.5	0.07	0.77	0.68
MRC	2 ± 0.69	2 ± 0.61	0.49	2.3 ± 0.69	2 ± 0.81	0.49	0.72	0.56
Ashworth	2 ± 0.57	2 ± 0.55	0.13	2 ± 0.57	1.5 ± 0.44	0.64	0.44	0.78
24 weeks								
FM	38.2 ± 9.7	35.6 ± 8.5	0.38	38.8 ± 9.7	36.6 ± 7.4	0.36	0.78	0.48
mBI	74.8 ± 11.5	68.4 ± 9.3	0.05	75.85 ± 11.5	72.5 ± 8.89	0.05	0.59	0.71
MRC	3 ± 0.82	2.5 ± 0.73	0.31	3 ± 0.82	3 ± 0.75	0.30	0.71	0.43
Ashworth	2 ± 0.66	2 ± 0.50	0.40	1.5 ± 0.66	1.5 ± 0.57	0.43	0.73	0.58

Exp 1: mononuclear stem cell (MNC); Exp 2: mesenchymal stem cell (MSC); Experimental: (MNC + MSC).

Table 3
Comparison of BOLD activation between stem cell and control groups.

Cluster	Z score	MNI coordinates (x, y, z) mm	Hemisphere	Area of activation	Brodmann area
165	4.49	−30 34 66	Right cerebrum	Precentral gyrus	Brodmann area 4
203	4.32	−32 38 60	Right cerebrum	Middle frontal gyrus	Brodmann area 6
121	4.67	−20 −22 40	Right cerebrum	Inferior parietal lobule	Brodmann area 40
74	3.90	−2 26 24	Left cerebrum	Anterior cingulate	Brodmann area 24
30	2.78	8 −58 6	Right cerebrum	Lingual gyrus	Brodmann area 18

weeks and 24 weeks. We observed an increased number of cluster counts in the Brodmann areas of patients administered with stem cell therapy as compared to those who were given physiotherapy regime only (Fig. 2). It was found that right Brodmann area 4, 6 had cluster counts of 165 and 203 respectively as compared to the control group (Table 3). Vigorous physiotherapy regime led to activation of inferior parietal lobule (BA 40) which is mirror neuron and learning area in brain.

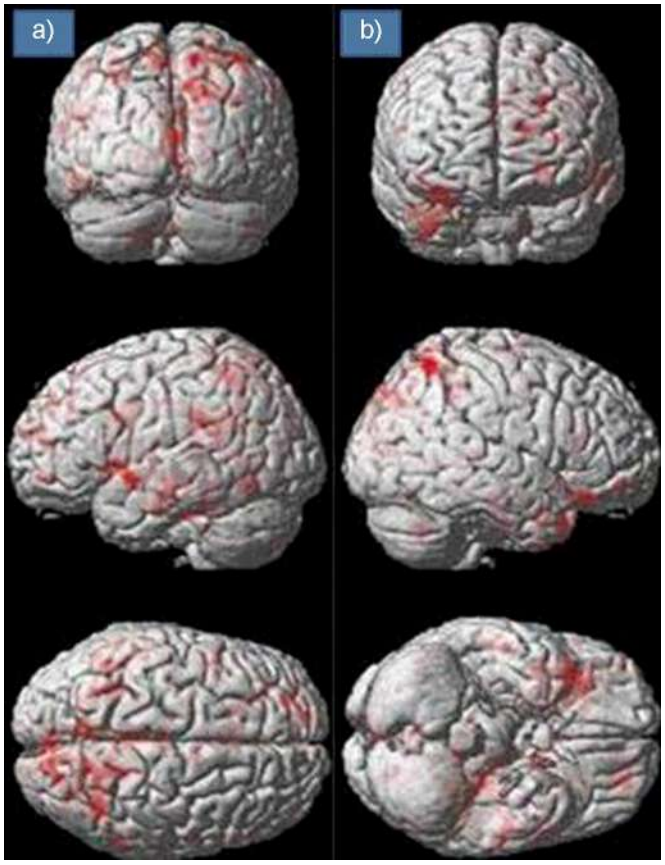

Fig. 2. BOLD activation in stem cell group (a) with respect to controls (b) overlaid on rendered images. Increased activation was observed in primary, premotor and parietal areas in stem cells group as compared to controls.

3.4. Comparison between mononuclear (exp 1) and mesenchymal (exp 2) stem cell groups

All clinical scores i.e., FM, Ashworth tone scale, MRC and mBI were statistically insignificant at 8 wks and follow up between the two experimental sub groups ($p > 0.05$) as shown in Table 2. In functional imaging analysis, both the groups had similar brain activation on BOLD imaging and neither of the group showed better results. When BOLD results of MNC/exp 1 group was compared with MSC/exp 2 group, it was found that right BA 6 had 63 voxels active, left BA 38 had 95 voxels active (Fig. 3). When MSC group was compared with MNC group, MSC group who had an activation of right dorsal premotor cortex (medial frontal gyrus), BA 6 with cluster counts of 44 voxels and left BA 40 (inferior parietal lobule) with cluster counts of 25 voxels (Table 4).

4. Discussion

Autologous intravenous stem cell therapy is safe and feasible to be transplanted in stroke subjects as shown by our results. We used the naïve i.e., mononuclear and culture expanded i.e., mesenchymal stem cells and tried to evaluate the safety, feasibility and efficacy of the two types of cells. We were able to procure 50–60 million mononuclear stem cells from the given bone marrow in 1–2 h of bone marrow aspiration whereas mesenchymal stem cells were obtained at mean fourth passage of fourth week of aspiration. No immunosuppressants were required following transplantation eliminating the risks associated with MSC therapy. The clinical, laboratory and radiological evaluations did not report any deaths, cell related complications or stroke recurrence. We used serum free media for the mesenchymal stem cell expansion unlike bovine serum used in the earlier study [21].

The mean percentage change in FM scores from baseline to 8 weeks and baseline to 24 weeks was 66.6% and 102.2% respectively in the stem cell group and 55.8% and 88.6% in the control arm

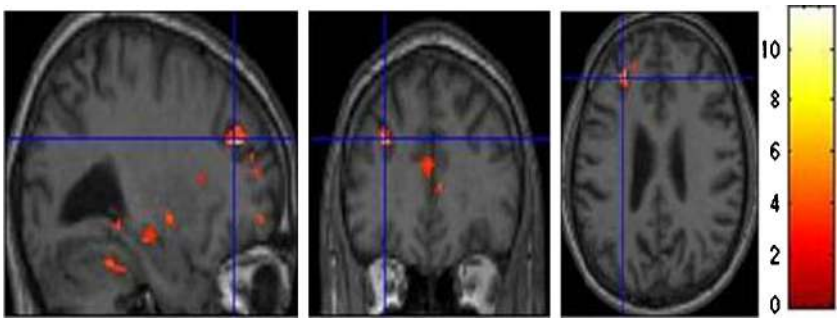

Fig. 3. Increased BOLD activation in MNC group with respect to MSC group overlaid on anatomical images.

Table 4
Comparison of BOLD activation between exp 2 and exp 1 groups.

Cluster	*Z* score	MNI coordinates (*x*, *y*, *z*) mm	Hemisphere	Area of activation	Brodmann area
25	3.45	−40 0 −6	Left cerebrum	Superior temporal gyrus	Brodmann area 40
44	3.76	−32 24 66	Right cerebrum	Medial frontal gyrus	Brodmann area 6
179	3.70	−22 −58 0	Left cerebrum	Lingual gyrus	Brodmann area 19
19	3.63	−46 −20 −2	Right cerebrum	Superior temporal gyrus	Brodmann area 22
16	3.62	−22 −44 8	Left cerebrum	Parahippocampal gyrus	Brodmann area 30
40	3.38	12 −60 4	Right cerebrum	Lingual gyrus	Brodmann area 18
48	3.18	−8 38 18	Left cerebrum	Anterior cingulate	Brodmann area 32

respectively. These figures suggest that there was a trend toward additional improvement in patients administered with stem cell therapy as compared to who were administered with physiotherapy regime only. We also observed that the modified Barthel Index scale was statistically significant at 24 weeks indicating role of stem cells in neural repair and recovery. When the two types of stem cells were analyzed individually it was found that patients with mononuclear stem cell administration improved significantly ($p < 0.05$) on activities of daily living scale i.e., Barthel Index as compared to the control group suggesting that mononuclear stem cells help in functional recovery as explained in previous trials. The same was not observed with the culture expanded cells i.e., mesenchymal stem cells where the results on all outcome measures were statistically insignificant ($p > 0.05$). This could be due to the sample size of mesenchymal patients ($n = 6$) as compared to mononuclear stem cells ($n = 14$) which might have altered the results. There was no statistically significant ($p > 0.05$) difference in the FM, Ashworth, MRC, LI and FA ratios when the two groups were compared.

The earlier studies received 50 million cells twice [22], 200–400 million cells [23], 34.6 million cells and 5–10 million cells [24,25]. We transplanted 50–60 million cells which was in congruence with these studies. There were no adverse reactions, mortality or any other risk factors involved with stem cell administration with the mentioned dose. Cell-enhanced recovery has been reported with chronic delivery of cells even at 1 month after ischemia. The best route of transplantation still needs to be established considering the specific cell type or the mechanism of action underlying the beneficial effect [26–28].

We observed that there was larger activation in supplementary motor cortex (BA 6) than the primary execution area (BA 4) at baseline, in nearly all patients suggesting recruitment principle of plasticity [29,30]. After physiotherapeutic regime for 8 weeks, it was observed that there was an increased number of voxels in BA 6 in both the groups. At follow up in the stem cell group, it was observed that primary hand motor area (BA 4) had increased number of voxels (focussing principle of neural plasticity) along with the premotor and supplementary motor areas (BA 6) compared to control group indicating the lasting effects of stem cells at 6 months (Fig. 2). In both the groups, we noticed that there was a considerable increase in LI and signal intensity when measured from baseline to 8 weeks indicating that a focused exercise regime which involves vigorous training of the hand using motor imagery principles, led to an increased force of activation performing better in the activities of daily living and a higher parietal, premotor and primary motor cortical activation [31].

The hypothesis to transplant stem cells was not only that the cells when given intravenously will preferentially home in the lesioned brain but also that these cells secrete neurotrophic growth factors acting as “scaffolds” making the host environment conducive for behavioral recovery in the form of ‘learning’ [32].

The optimal dosage of cell therapy is still under research. It has been suggested that 1–2 million cells/kg of body weight is sufficient to study functional recovery. The optimal time to transplantation after a stroke is still unknown. The brain environment changes dramatically over time after ischemia. In the acute phase there is an increase in excitatory amino acid release, peri-infarct depolarization, and reactive oxygen species release. This is followed by an inflammatory/immune response and cell death, which, in the penumbra, can last up to several weeks. Brain repair and plasticity after the acute phase take place over several weeks to months. If a treatment strategy focuses on neuroprotective mechanisms, acute delivery of the cells will be critical. If the cells act to enhance endogenous repair mechanisms (e.g., plasticity, angiogenesis, and neurogenesis) or require events in order to survive and integrate, then early delivery would be pertinent because these events are most prevalent in the first 2–3 weeks after ischemia. If cell survival is important, then transplanting late, after inflammation has subsided, could be beneficial.

From a small sample sized, non randomized study, we could not reach a successful conclusion regarding the efficacy of cells as to which type is better than the other but we managed to show a trend of improvement in stroke patients. The study might have yielded different results according to the etiology and time of event. But the sample size is so small that to categorize the results according to etiology may not have been significant to report. As this was primarily a safety and feasibility study we selected patients according to functional and impairment status, wherein the spontaneous recovery has already traded off and who do not have residual permanent disability and handicap (3 months to 2 years). Indeed two clinical trials have chosen to study patients at least 6 months post stroke (Savitz et al. [33]). We did not observe any infection, bleeding, edema, thrombus formation, tumorogenesis, ectopic

tissue formation, cardiovascular & neurological deterioration or any behavioral abnormality up to six months. A longer follow up would suggest the effectiveness of stem cell therapy to be used in stroke patients. Other factors in designing a trial would be dose of cells, site and mode of transplantation and recovery factors post stroke [34,35]. An interim analysis of the same study has been published indicating the safety of the stem pro serum free media used for ex vivo culturing of cells [36].

Stroke initiates activation of self-repair mechanisms comprising plastic changes at the synaptic level, reorganization of existing and establishment of new neuronal circuits, and cell genesis, all contribute to recovery of a stroke injured brain [37]. Intervention by stem cells may not have replaced the damaged neurons but may have acted through other endogenous mechanisms/neurotrophic therapy which might have led to important implications for recovery [38,39].

5. Conclusion

Cell transplantation therapy presents third wave of stroke therapeutics. The fact that substantial functional gains have been seen in preclinical stroke models is encouraging yet bench to bed side work emphasize on safety and feasibility of cells. This study primarily redefines the safety, tolerance and feasibility of bone marrow derived stem cells in chronic stroke. More studies are needed to ensure high standards of safety and efficacy of Stem Cell therapy for clinical trials.

Source of funding

This research has been funded by Department of Science and Technology, New Delhi, India.

Conflicts of interest

None declared.

Acknowledgements

All the authors have contributed significantly in this research.

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