Autologous Intravenous Mononuclear Stem Cell Therapy in Chronic Ischemic Stroke

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Background: The regenerative potential of brain has led to emerging therapies that can cure clinico-motor deficits after neurological diseases. Bone marrow mononuclear cell therapy is a great hope to mankind as these cells are feasible, multipotent and aid in neurofunctional gains in Stroke patients.

Aims: This study evaluates safety, feasibility and efficacy of autologous mononuclear (MNC) stem cell transplantation in patients with chronic ischemic stroke (CIS) using clinical scores and functional imaging (fMRI and DTI).

Design: Non-randomised controlled observational study

Study: Twenty-four (n=24) CIS patients were recruited with the inclusion criteria as: 3 months–2 years of stroke onset, hand muscle power (MRC grade) at least 2; Brunnstrom stage of recovery: II-IV; NIHSS of 4-15, comprehensible. Fugl Meyer, modified Barthel Index (mBI) and functional imaging parameters were used for assessment at baseline, 8 weeks and at 24 weeks. Twelve patients were administered with mean 54.6 million cells intravenously followed by 8 weeks of physiotherapy. Twelve patients served as controls. All patients were followed up at 24 weeks.

Outcomes: The laboratory and radiological outcome measures were within normal limits in MNC group. Only mBI showed statistically significant improvement at 24 weeks (p<0.05) whereas the mean FM, MRC, Ashworth tone scores in the MNC group were high as compared to control group. There was an increased number of cluster activation of Brodmann areas BA 4, BA 6 post stem cell infusion compared to controls indicating neural plasticity. Cell therapy is safe and feasible which may facilitate restoration of function in CIS.

Key Words: Cell transplantation, Stroke, Mononuclear Stem Cells, Functional Recovery, Neuroimaging

Introduction

The frightening incidence of stroke in society has fuelled interest in regenerative medicine to repair brain after an acute insult or restore maximum functionality in the shortest possible time. It is also a well-known fact that more than half of stroke survivors are dependent on caregivers. These findings have led the researchers to develop newer therapies and cell transplantation is one such hope which holds a great promise. Two reviews, six clinical trials and one case report have been published with stem cell therapy in ischemic and hemorrhagic stroke. Embryonic, fetal neural, immortalised cell lines, human umbilical cord blood and bone marrow derived cells have been exploited in stroke, aiding in recovery through secretion of neurotrophic factors, promoting endogenous repair and preventing apoptosis.

Hematopoietic stem cells (HSC)

It has been postulated in rat models and clinical trials that bone marrow mononuclear stem cells are capable of neurogenesis and angiogenesis modulating the ‘host environment’. Mononuclear stem cells have been experimented in preclinical studies suggesting increased angiogenesis in penumbral tissue following CD34+ cell transplantation, whether given systemically or by the intracerebral route. The aforementioned reports demonstrated a good functional recovery, as well as reduction in infarct size. A number of clinical trials are currently underway investigating the role of bone-marrow-derived mononuclear stem cell therapy at different stages of ischemic stroke and utilising different methods of delivery but the results of same are awaited.

Functional Recovery in stroke

Recovery in stroke has been widely explained by neural plasticity. Spontaneous recovery occurs within 3 months of an insult followed by a plateau phase which can be remodelled by external and internal inputs. Longitudinal studies of recovery after stroke suggest that only 50% of patients with significant arm paresis recover useful hand function. Injury-related and use-dependent cortico-motor reorganization has been extensively studied as a recovery process following stroke. Functional imaging provides an excellent information about neurobiology at cellular and molecular levels that occur post stroke by acting as surrogate markers providing insights into spontaneous and therapy induced recovery after stroke. Blood Oxygen Level Dependent (BOLD) and diffusion tensor imaging (DTI) techniques predict the functionality of brain areas and evaluate integrity of white matter tracts i.e., corticospinal tracts (CST) optimising the motor recovery potential.
Cell transplantation as a neurorestorative therapy

The exact timing for cell transplantation has not been well defined because of the dynamic modifications of the ischemic lesion’s environment. Cell transplantation in the acute phase of stroke has been thought to encounter difficulties due to the hostile lesion environment generated by the acute inflammatory agents i.e., cytokines. Some studies have reported an increased synaptophysin expression in the penumbral tissue after intravenous administration of human bone marrow stromal cells. Thus it is advisable to delay transplantation until neurological deficits reach a plateau where any further spontaneous recovery is unlikely.

The principle of restorative therapies is based on the concept of reorganizing brain, promoting implicit learning in the cortex with the lesion. Initially, it was thought that cell therapy might work by ‘cell replacement’ mechanism, however recently a good amount of evidence has emerged suggesting that cell therapy works by providing trophic or ‘chaperone’ support to the injured tissue and brain.

The present stem cell research studies the safety, feasibility and efficacy of intravenous autologous mononuclear stem cells in chronic ischemic stroke. It also studies cortical reorganisation by functional imaging techniques i.e., BOLD and DTI when patients were administered with mononuclear stem cell therapy with physiotherapy regime for 8 weeks.

Methods

Patients diagnosed with ischemic stroke from 3 months to 2 years of index event were recruited with MRC (Medical Research Council) grade of power for wrist and hand flexor / extensor muscles of at least 2. Brunnstrom stage of recovery: II-IV, NIHSS (National Institute of health stroke scale) of between 4 and 15, conscious and comprehensible. The exclusion criteria were autoimmune disorders, immune-compromised subjects, chronic liver and renal failure, hematological disorders, progressive neurological worsening, unilateral neglect, neoplasia, contraindication to MRI and pregnancy.

This study is a non randomized controlled clinical trial. The trial protocol and informed consent form were approved by the IC SCRT (Institute committee for stem cell research and therapy) and it is registered with CTRI.

Procedures

Bone marrow aspiration, separation and transplantation

Bone marrow (approx 40-50 ml) was aspirated under aseptic conditions from the posterior superior iliac crest in twelve (n=12) chronic ischemic stroke patients. Bone marrow aspirate was diluted with phosphate buffered saline, layered over ficoll density medium and centrifuged at 1800 rpm for 25 minutes. Bone marrow mononuclear cells (BM-MNC) layer was collected, their sterility and viability was maintained before transplantation. MNC count and number of CD 34+ cells (flow cytometry method) were counted for each patient. The whole procedure took approximately 120 ± 10 minutes, i.e., approximately 2 hours[17].

| No | Age | Months after Stroke | Risk factors | Type of stroke | Area of Infarct | Volume of lesion (ml) | No of cells | %CD 34+ cells | Baseline FM (I/66) | 8 weeks FM (I/66) | 24 weeks FM (I/66) | Baseline mBI (I/100) | 8 weeks mBI (I/100) | 24 weeks mBI (I/100) |
|----|-----|---------------------|--------------|---------------|----------------|----------------------|-------------|--------------|-----------------|----------------|-----------------|----------------|----------------|----------------|
| 1  | 32/M| 4                   | Smoking, alcohol | LA | Lt fronto parietal | 11.4 | 56 x 10^6 | 0.2 | 22 | 52 | 40 | 70 | 48 | 82 |
| 2  | 62/M| 8                   | Dyslipidemia, HTN, | LA | Rt frontal | 25.6 | 54 x 10^6 | 0.10 | 14 | 32 | 20 | 53 | 28 | 65 |
| 3  | 49/M| 7                   | HTN, DM, dyslipidemia | LA | Rt fronto parietal | 22.5 | 50 x 10^6 | 0.10 | 14 | 43 | 21 | 72 | 28 | 84 |
| 4  | 42/M| 12                  | DM, alcohol | SV | Rt temporal | 8.2 | 58 x 10^6 | 0.1 | 24 | 52 | 42 | 80 | 54 | 90 |
| 5  | 55/M| 15                  | HTN, DM | SV | Rt fronto parietal | 7.8 | 57 x 10^6 | 0.2 | 22 | 50 | 39 | 65 | 44 | 72 |
| 6  | 60/M| 7                   | HTN | LA | Rt striato capsular | 18.4 | 55 x 10^6 | 0.7 | 17 | 50 | 32 | 76 | 40 | 85 |
| 7  | 42/M| 8                   | Smoking | LA | Lt PLIC | 17.8 | 56 x 10^6 | 0.7 | 22 | 58 | 34 | 70 | 38 | 78 |
| 8  | 35/M| 14                  | Alcohol, smoking | SV | Lt | 6.2 | 58 x 10^6 | 0.028 | 44 | 72 | 54 | 92 | 60 | 98 |
| 9  | 40/M| 6                   | smoking | DT | Rt striato capsular | 9.3 | 55 x 10^6 | 0.730 | 22 | 55 | 43 | 73 | 49 | 84 |
| 10 | 32/F| 8                   | - | UN | Lt fronto/ parietal | 34 | 52 x 10^6 | 0.20 | 12 | 40 | 23 | 52 | 28 | 60 |
| 11 | 50/M| 11                  | HTN | DT | Rt parietal | 11.4 | 53 x 10^6 | 0.40 | 17 | 43 | 32 | 72 | 39 | 82 |
| 12 | 60/M| 12                  | HTN, DM, smoking | LA | Lt fronto temporal/ parietal | 43 | 52 x 10^6 | 0.20 | 11 | 38 | 22 | 50 | 28 | 64 |
| Mean | 46.5 | 9.3 | | | | 17.9 | 54.6 x 10^6 | 0.30 | 20.08 | 48.75 | 33.5 | 68.75 | 40.33 | 78.6 |

HTN: Hypertension; DM : diabetes mellitus; LA: large artery; SV : small vessel; CE : cardioembolic; DT : stroke with determined etiology; UN: stroke with undetermined etiology

Table 1. Demographic and clinical data in the stem cell / MNC group at baseline, 8 weeks and 24 weeks
were transplanted with mononuclear stem cells the same day of expansion. 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. An aseptic technique of infusion was performed where the cells were given in a sterile 50 ml syringe which was directly dissolved in 250 ml of saline bottle and infused intravenously over three hours.

Method of selection

Although it was a non-randomized trial, it was made sure that the patient selection for stem cells was unbiased and the two groups were matched according to the age, severity, time of onset of stroke and lesion size. All the patients were examined by a neurologist and a neuro-physiotherapist for strength, tone (modified Ashworth), Brunnstrom stage of stroke recovery, Fugl Meyer (FM) scale for upper limb and modified Barthel index (mBI) at baseline, 8 weeks and at 24 weeks of stem cell transplantation [26-27] along with functional MRI (BOLD and DTI). We used Edinburgh handedness inventory to assess hand dominance. A neurologist helped in treatment allocation but was not a part of the study. The radiological and imaging variables were blinded by a one of the authors. The physiotherapy intervention was based on the concept of mirror therapy and motor imagery [29 30]. The treatment was administered by a qualified physiotherapist for 5 days/week for 8 weeks for 80-90 minutes.

Functional MRI and acquisition

Subjects were asked to perform the motor task with affected hand, with self-paced (minimum 0.5Hz) fist clenching/extension of the wrist or metacarpophalangeal (MCP) joints of the hand depending upon the extent of motor damage. To have consistency of rate of motion and to reduce artefacts, the movement was visually monitored using a remote digital camera. Blood oxygenation level dependent (BOLD) data was acquired using the gradient echo planar imaging (EPI) sequence using 1.5T MR scanner (Avanto, M/s. Siemens Medical Solutions, Germany) with a standard head coil. Block design with alternate baseline and activation cycles was used with a total of 90 whole brain EPI measurements with (TR=4520ms, TE=44ms, slices=31, slice thickness=4 mm) after a dummy scan of 2 measurements with same parameters and MPrage sequence with 176 contiguous slices of 1.0 mm thickness [31].

Diffusion Tensor Imaging

Diffusion tensor images were acquired with single shot echo planar technique with b values 0, 400, 1000 s/mm² in twenty directions, 128x128 matrix; field of view: 250 x 250 mm, TE = 76 ms, TR = 10,726 ms, Echo planar imaging (EPI) factor = 127, slice thickness of 2.3 mm. The termination criteria used were Fractional anisotropy (FA) < 0.2 with an angle change >45°. Seed points as region of interest (ROI) were drawn in the infarcted area and corticospinal tract (CST) portion in the affected and unaffected hemisphere. The selection of ROIs was repeated thrice by one rater and the average value was regarded as the unit of measurement. Fiber tracts passing through these ROIs were designated as the final tracts of interest. The fractional anisotropy (FA) indices and apparent diffusion co-efficient (ADC) values were calculated in the drawn ROIs.

Post-processing

FMRI data were analysed using SPM2 software under MATLAB environment (Mathworks). The functional images were realigned, normalised and then smoothened by a 6-mm Gaussian filter. Design matrix was created with MNI (Montreal Neurological Institute) co-ordinates with the number of cluster counts activated. These MNI co-ordinates were correlated with Talairach’s atlas to get the anatomical location in the brain. The volume of the lesion was analysed using J-IMAGE software (NIH).

Statistics

The statistical analysis was done by SPSS 11.5. We used parametric tests for FM and mBI scores and non-parametric tests for functional MRI analysis between groups. We used paired t-test for intra group and independent sample t-test for inter group comparisons with p=0.05. Repeated measures ANOVA was used to calculate the difference at baseline, 8 weeks and at 24 weeks.

Results

Twelve patients were administered with bone marrow derived mononuclear stem cells. All the patients had ischemic stroke in the mononuclear stem cell (MNC) group with seven patients (n=7) with right hemisphere lesion and five patients (n=5) with left hemisphere lesion.

Safety of stem cell transplantation

The routine laboratory tests at 1st, 3rd and 7th day of transplantation were performed which were within normal limits for all patients in the stem cell group. The cells had an average viability of 99% before transplantation. The transplanted cells were unfractonated mononuclear stem cells but CD 34+ was quantified to evaluate the proportion of these cells in the implanted volume. The mean CD 34+ count was 0.304% with mean 54.6 x 10⁶ MNC (table 1) diluted in 250 ml of saline, infused intravenously in the arm vein. There were no major immediate or delayed adverse reactions observed at the time of bone marrow aspiration, during and after MNC transplantation. Two of the patients reported pain at the site of bone marrow aspiration which was treated. We did not observe any infection, bleeding, edema, thrombus formation, cardiovascular and neurological deterioration up to six months.

Clinical results

The demographic data, the time of stroke onset and the lesion site along with the clinical scores of Fugl Meyer scale (FM) and modified Barthel index (mBI) in stem cell and control group are shown in table 1 and 2 respectively. In the mononuclear stem cell group (M:F=11:1), (mean age ± SD = 46.5 ± 10.9), the mean baseline FM score was 20.08 ± 8.8 and at 8 weeks was 33.56 ±10.62, showing statistically significant improvement using paired t-test (p = 0.0001, t= - 9.93). The follow up at 24 weeks had a mean FM of 40.33 which was higher than the mean at 8 weeks in these patients exhibiting statistical significant improvement (p=0.0001,
Table 2. Demographic and clinical data in the control group at baseline, 8 weeks and 24 weeks.

| No | Age/ sex | Months after Stroke | Risk factors | Type of stroke | Area of Infarct | Volume of lesion (ml) | Baseline FM (mm) | 8 weeks FM (mm) | 24 weeks FM (mm) | Baseline mBI | 8 weeks mBI | 24 weeks mBI |
|----|----------|---------------------|--------------|----------------|----------------|----------------------|-----------------|----------------|----------------|-------------|-------------|-------------|
| 1  | 62/M     | 9                   | HTN, DM, Smoking, dyslipidemia | LA | Rt parietal | 11.2 | 14 | 55 | 26 | 65 | 32 | 74 |
| 2  | 50/M     | 3                   | Dyslipidemia, DM HTN, alcohol | LA | Lt parietal | 15.4 | 17 | 43 | 30 | 55 | 36 | 64 |
| 3  | 45/M     | 4                   | HTN, DM | LA | Rt caudate | 9.8 | 26 | 52 | 34 | 61 | 34 | 73 |
| 4  | 43/M     | 13                  | DM, Smoking | LA | Lt parietal | 7.8 | 22 | 50 | 37 | 61 | 44 | 70 |
| 5  | 58/M     | 10                  | HTN, DM, smoking, dyslipidemia | LA | Lt parietal | 21.2 | 14 | 32 | 21 | 43 | 28 | 57 |
| 6  | 55/M     | 5                   | HTN, DM, Smoking, dyslipidemia | LA | Lt striato capsular / parietal | 9.3 | 22 | 58 | 32 | 69 | 36 | 79 |
| 7  | 60/M     | 9                   | HTN, dyslipidemia, Smoking | LA | Rt temporoparietal | 7.1 | 24 | 52 | 39 | 62 | 44 | 72 |
| 8  | 40/M     | 4                   | Smoking | SV | Lt caudate | 8.8 | 22 | 52 | 37 | 60 | 44 | 72 |
| 9  | 40/M     | 7                   | Smoking | SV | Rt frontal | 5.8 | 42 | 72 | 52 | 80 | 58 | 90 |
| 10 | 45/M     | 9                   | HTN | LA | Rt int. capsule | 15.7 | 24 | 50 | 40 | 60 | 48 | 72 |
| 11 | 32/F     | 13                  | - | CE | Lt frontoparietal | 21.2 | 12 | 40 | 21 | 50 | 28 | 54 |
| 12 | 35/M     | 14                  | - | CE | Lt parietal | 34.6 | 14 | 43 | 20 | 52 | 26 | 60 |
| Mean | 47.0 | 8.3                  | - | - | - | 13.9 | 21.08 | 49.9 | 32.41 | 59.8 | 38.1 | 69.7 |

HTN: Hypertension; DM: diabetes mellitus; LA: large artery; SV: small vessel; CE: cardioembolic; DT: stroke with determined etiology; UN: stroke with undetermined etiology.

Repeated measures ANOVA was used to compare baseline, 8 weeks and 24 weeks scores and was found to be statistically significant (p<0.05). The mean mBI in the stem cell group at baseline, 8 weeks and 24 weeks was 48.75 ± 10.56, 68.75 ± 12.27 and 78.6 ± 11.34 respectively showing statistical significant improvement (p<0.05). The male to female ratio in the control group was 10:2, with mean age ± SD =47.08 ± 9.9. The mean baseline FM score was 21.08 ± 8.15 and at 8 weeks was 32.41 ± 9.49 showing statistically significant improvement (p<0.05). These patients also showed statistically significant improvement for mBI scores between baseline and 8 weeks and between (8-24) weeks (p<0.05).

Functional MRI results

BOLD activation was observed in the primary motor cortex (BA 4), precentral gyrus, supplementary motor area, premotor area (BA 6), inferior temporal gyri and cerebellum. Since there was variability in the number of active voxels in the brain, we calculated the laterality index (LI) of the contralesional/ ipsilesional hemisphere. The LI of ipsilesional BA 4 and BA 6 showed statistically significant improvement when measured between baseline and 8 weeks (p=0.005 and p=0.02 respectively) (table 3). Similar results were observed in the control group (p=0.04 and p=0.05 respectively). Both the results were non-significant when measured between 8 and 24 weeks (p=0.208 and p=0.225 respectively).

The Fractional Anisotropy (FA) ratio was calculated as FA of the affected hemisphere (ah) divided by the unaffected hemisphere (uh) i.e., (FA_{ah}/FA_{uh}). The FA values of the affected hemisphere were lower as compared to the unaffected hemisphere in both the groups. These values correlated well with the Fugl Meyer score, with a lower FA having a low FM and higher FA (> 0.6) with a higher FM score predicting good motor recovery (Steiner, Barber., et al 2007). We also calculated the number of fibres and fibre length in the given ROI of affected and unaffected hemispheres. We calculated the fibre length ratio of affected hemisphere to unaffected hemisphere (FL_{ah}/FL_{uh}) and the ratio of the fibre number (FN_{ah}/FN_{uh}). In the stem cell / experimental group, the mean fibre length (mm) in the affected hemisphere at 0 week was 19.46 ± 15.86, at 8 weeks was 21.70 ± 17.46 and at 24 weeks was 27.51 ± 21.58, whereas in the unaffected, it was 64 ± 12.64, 65.30 ± 12.7 and 66.23 ± 12.01 respectively. The mean FL ratio was 0.30, 0.33 and 0.41 at 0, 8 and 24 weeks respectively. There was a change of 0.03 mm from baseline to 8 weeks and a change of 0.11mm from baseline till 24 weeks in this group. In the control group, the mean fibre length in the affected hemisphere at 0 week was 20.96 ± 15.86, at 8 weeks was 23.22 ± 16.68 and at 24 weeks was 26.18 ± 18.09. The FL ratio was 0.31 at 0 weeks, 0.34 at 8 weeks and 0.38 at 24 weeks. There was change of 0.03 mm from baseline to 8 weeks and 0.07 mm from baseline till 24 weeks in this group. The mean fibre number ratio (FN_{ah}/FN_{uh}) in the stem cell was 0.30, 0.402 and 0.49 at baseline, 8 weeks and at 24 weeks. There was no significant improvement in the number of fibres after stem cell therapy. The mean fibre ratio in the control group was 0.30, 0.39 and 0.49 respectively.
Between group comparisons

Two sample t-test was applied for inter group comparison and there was no significant difference observed in Fugl Meyer score at 8 weeks and 24 weeks (p = 0.79 and p = 0.61 respectively) (figure 1) but mBI showed statistically significant improvement in the MNC/ stem cell group as compared to control group (p = 0.058 at 8 weeks and p =0.026 at 24 weeks) (figure 2).

| Subject id | Task        | BASELINE | 8 WEEKS | 24 WEEKS |
|------------|-------------|----------|---------|----------|
|            |             | BA4 L    | BA6 L   | FA        | Ips CB activ. ratio | FA | Ips CB activ. ratio | FA | Ips CB activ. ratio |
| 1          | RFM         | -922     | -1      | 196      | 100     | 0.32 | 0.38 | 0.57 | 0.156 | 0.132 | 0.09 | 0.698 | 0.516 | 0.38 | 0.4 | 0.61 |
| 2          | LFM         | - -      | -150    | -1      | 0.32    | 0.09 | 1   | - | 1510 | 280 | 1510 | 0.74 | 0.4 | 0.15 |
| 3          | LFM         | 120      | 614     | 0.23    | 932     | 141  | -0.8 | 0.56 | 0.38 | 0.48 | - | 1159 | 99 | 700 | 0.82 | 0.56 | 0.41 |
| 4          | LFM         | - -      | 1293    | 1293    | 0      | 0.81 | 0.35 | 0.13 | - | - | - | 2076 | 1 | 0.81 | 0.42 |
| 5          | LFM         | 313      | 230     | 0.32    | 313     | 212  | 0.19 | 0.49 | 0.15 | 1 | 163 | 831 | 0.66 | 1899 | 4101 | 0.85 | 0.49 | 0.2 |
| 6          | LFM         | 112      | 287     | 0.43    | 99      | 890  | 0.79 | 0.42 | 0.38 | 1 | - | - | 76 | 1729 | 0.91 | 0.52 | 0.4 |
| 7          | RFM         | 82       | 1 | 122 | - | 1 | 0.62 | 0.35 | 1 | 230 | - | 1 | 512 | - | 1 | 0.72 | 0.5 |
| 8          | RFM         | 52       | 153     | -0.49   | 27      | 484  | -0.92 | 0.82 | 0.59 | - | 376 | 76 | 0.67 | 1570 | 20 | 0.97 | 0.89 | 0.81 |
| 9          | LFM         | 62       | 1 | - | 261 | 1 | 0.52 | 0.33 | 0.34 | - | 123 | 1 | - | 1463 | 1 | 0.67 | 0.45 |
| 10         | RFM         | -        | 147     | 89 | 0.24 | 0.46 | 0.1 | 1 | 596 | 412 | 0.18 | 419 | 133 | 0.34 | 0.46 | 0.26 |
| 11         | LFM         | 230      | 560     | 0.56    | 400     | 690  | 0.67 | 0.67 | 0.29 | 220 | 789 | 0.98 | 230 | 1020 | 0.9 | 0.72 | 0.5 |
| 12         | RFM         | 56       | 643     | -0.98   | 120     | 450  | -0.34 | 0.38 | 0.04 | - | 450 | 340 | 0.33 | 670 | - | 1 | 0.38 | 0.05 |

Table 3. BOLD brain activation pattern and voxel counts in BA 4, BA 6 and cerebellum in the mononuclear stem cell group.
The Laterality index of ipsilesional BA 4 and 6 between stem cell and control group showed a statistically significant difference (Kruskal wallis test) at 8 weeks (p= 0.0001 for BA 4 and p= 0.003 for BA 6) but was non-significant at 24 weeks. There was no significant change in the FA ratio, fiber length (FL) ratio and fiber number (FN) ratio between the two groups at 8 weeks and 24 weeks.

We compared the BOLD activation of both right and left hemispheric lesion stroke in the stem cell/ MNC group with the control group at 8 weeks and at follow up. When the right hemispheric lesion stroke was compared with the control group patients, it was found that the premotor cortex of the affected (right) hemisphere were active with a cluster count of 199 voxels along with the inferior parietal lobe of both the hemispheres at 8 weeks. At 24 weeks, there were 150 voxels active in right BA 6 along with limbic and frontal lobe areas as compared to the control group (table 4) (figure 3). In the left hemispheric lesion stroke, it was found that ipsilesional primary and premotor cortices were active along with the areas involved in the activation of mirror neurons. The results were similar at 24 weeks with cluster activation 258 voxels and 110 of BA 6 and BA 40 each respectively (data not shown).

### Discussion

The mononuclear stem cells were easy to procure from the bone marrow and had an average viability of 99% before transplantation which is similar to what is described in previous trials [9, 10, 11]. Data from Kondziolka et al [5] showed that stem cell transplantation was no better or worse at reducing the risk of death, compared to routine treatment alone (95% CI 0.01 to 2.31, p=0.16). Our patients with stem cell transplantation did not report neurological worsening or any new chief complaints during the time they were followed up. The laboratory reports were normal and radiological evaluation did not show any new structural abnormality or stroke re-occurrence. Thus the safety, feasibility and tolerance of autologous MNC transplantation in stroke patients were established.

Our study has similar findings with other published trials having no adverse reactions, mortality or any other risk factors involved with MNC administration of up to 50-60 million cells in chronic stroke patients. The dose of stem cells prescribed in our study was in congruence with the previous trials and the current ongoing studies. [18, 19] The earlier intervention groups received 50 million cells twice [8, 9], 200 million or 400 million cells once [5, 6] and 5 or 10 million cells once [9] in stroke patients. The recent study of BM MNC in stroke intralesionally transplanted mean 34.6 million cells in stroke patients. [10]

| Cluster | Z score | mni coordinates (x,y,z) mm | Hemisphere | Area of activation | Brodmann area |
|---------|---------|-----------------------------|------------|-------------------|---------------|
| 150     | 2.83    | -4 12 66                   | Right      | Medial frontal gyrus | Brodmann area 6 |
| 30      | 2.09    | -8 -34 4                   | Left       | Sub-lobar         |               |
| 14      | 2.49    | -22 -30 -4                 | Left       | Limbic Lobe       | BA 38         |
| 105     | 2.96    | 34 -20 38                  | Right      | Frontal Lobe      | BA 3          |

*One cluster 2*2*2 mm³*
The stem cells / MNC group had nine cortical and 3 sub cortical infarcts, with volume of lesion between (6.2–43 ml) and control arm had seven cortical and five sub cortical strokes with lesion volume (5.8–35 i.e 34.6 ml). All patients in both the groups improved significantly at 8 weeks and at follow up with an exceptional improvement in a patient (id 8) with a high FM=60 and mBI=98. To avoid ethical issues, we did not include a group without physiotherapy in the trial.

The clinical and functional outcomes that we used were Fugl Meyer (FM), modified Barthel Index (mBI), MRC grade for power, Ashworth tone grade scale and NIHSS (National Institute of Health Stroke scale). The previous trials had outcome measures as BI, FM, mRS, European Stroke Scale (ESS) and have observed statistically significant difference in BI at 3 and 6 months (p=0.011) [5, 6]. On comparing the stem cell with control group, it was observed that there was no difference in the FM score at 8 weeks (p>0.05) and at follow up of 24 weeks, but the mBI scores showed statistical significant improvement both at 8 weeks and 24 weeks (p < 0.05). In the stem cell group, the mean percentage change in FM score between (0-8 weeks) was 67.5% and between (8-25 weeks) was 21.19% and in the control arm was 52.4% and 17.7% respectively. The change from baseline (0 week) to 24 weeks in the MNC group was 95% as compared to 81% in the control arm. The above results suggested that mononuclear stem cells may have aided the behavioral and functional recovery as compared to the control group patients. We also observed reduction in pain, reduced spasticity in few patients and improvement in speech in two patients in the experimental group. However, we did not find a significant change in the power of the wrist and hand muscles.

The laterality index (LI) changed in few patients in both the groups i.e., patients (id 1,2,3 and 8) in the stem cell group and patients (id 4,5,9,12) in the control group (table 3). At baseline, we observed an increased activation in supplementary motor cortex (BA 6) than the primary execution area (BA 4) in some patients (recruitment principle of plasticity) (figure 4), irrespective of the hand cortical area being fully damaged / partially damaged or spared, but after physiotherapy regime for 8 weeks, it was observed that primary hand motor area had increased number of voxels (focussing principle of neural plasticity) [31, 32]. It was found that vigorous exercise training of the hand led to an increased force of activation, which was also reflected in the signal change of ipsilesional BA 4 (figure 3), and when the physiotherapy regime was withdrawn a decreased BOLD activation was observed which could be due to “learned non use”. We calculated ipsilateral cerebellum ratio to know the activity of cerebellum and observed that ipsilateral and contralateral cerebellum to the hand were active in all the patients stating its role in co-ordination, motor control and sequencing but we could not come to a conclusive evidence to state which sided cerebellum is active which is similar to the previous report. [33] The site and size of stroke lesion has been studied assessing functional MRI response in MCA stroke suggested that with recovery, greater recruitment was observed of the affected primary motor cortex (M1) and a decrease in activation of the unaffected M1 and supplementary motor area. In addition, the widespread activation of brain areas seen during the initial session changed to a more focused pattern of activation as the patient recovered. [34]

Our study reconfirmed the observation that FA index is a sensitive measure reflecting change in the integrity of the motor tracts, i.e., corticospinal tracts (CST) [35]. Lower FA ratio suggests deterioration of the CST leading to Wallerian degeneration. It was observed that nine (n=9) out of twelve patients (n=12) in the stem cell group showed good recovery at follow up with FA > 0.6 as compared to control group in which only seven out of twelve improved (figure 5).

We observed a small change in the fibre length ratio of 0.11mm in the stem cell group and 0.07 mm in the control group at follow up in comparison to 8 weeks. This may be due to recovery over time and other factors such dendritic sprouting and axonal remodelling hypothesized by the stem cell therapy [36] augmenting functional recovery. There was no change in the fibre number ratio (FN ratio) between the groups. The mean FN ratio at 0, 8 weeks and 24 weeks were 0.30, 0.40 and 0.49 at the determined ROIs in both the groups.

Motor imitation is a complex cognitive function that incorporates motor observation, imagery and execution which increases the excitability of the corticospinal pathway. It was observed that premotor cortex, dorsolateral prefrontal cortex and the primary hand motor area were highly active [30].

Figure 4. Activation maps superimposed on whole brain T1 images for (a-c) Pretreatment activation, (d-f) Post stem cell treatment activation in sagittal (a,d), coronal (b,e) and axial (c,f) orientations.

Figure 5. Correlation between FM scores and FA ratio in mononuclear stem cell (MNC) and control group at 24 weeks
We chose intravenous route as it is safer and feasible as compared to intrastratral. Studies have observed cell-enhanced recovery with chronic delivery of cells even at 1 month after ischemia and homing in to the infarcted regions. However, the best route of transplantation still needs to be established considering the specific cell type or the mechanism of action underlying the beneficial effect.

It is also postulated that stem cells operate not through a unidirectional mechanism i.e. forming neurons but rather as cellular mediators of many of biological mechanisms that could provide a favourable recovery in a neurological disease. Our aim to administer stem cells intravenously was not with the hypothesis only that stem cells will migrate preferentially to the injured cortex but more likely that the intravenously administered stem cells will help in up-regulation of growth factors within the body and brain making the host environment conducive for behavioral recovery in the form of ‘learning’. Enriched environment, physical activity, stress or molecules such as BDNF, VEGF and dextroamphetamine leads to reorganization of brain areas. We used autologous stem cells as 'stimulant' which enriched the host environment leading to cortical reorganization and clinical improvement. A definite conclusion regarding the efficacy of mononuclear stem cell transplantation intravenously cannot be drawn from our results and we acknowledge our limitations which were: small sample size, non-randomized trial, dose of cells, site of mode of transplantation, recovery factors post stroke and lack of in vivo monitoring of the intravenously transplanted cells. Nevertheless our results showed a high mean of FM, mBI, LI and FA ratios in the mononuclear stem cell group as compared to the patients who were administered with physiotherapy regime only.

Our study used BOLD contrast and diffusion tractography to study the functional recovery after stroke which can be a quantifiable outcome measure and is the first stem cell study using functional MR modalities. BOLD technique could study the neurobiological mechanisms of treatment i.e., the administration of autologous stem cells and physiotherapy. We studied cortical reorganization in stroke subjects by measurement of laterality index (LI), fractional anisotropy ratios (FA), signal intensity change of the activated hemisphere and the fibre tract density and length. More clinical trials are needed to validate the efficacy of stem cells.

Conclusion

Autologous bone marrow derived mononuclear stem cells are safe and feasible in chronic ischemic stroke. Cell therapy with physiotherapy regime led to a trend of improvement in clinical and fMRI scores at 8 weeks as compared to physiotherapy alone and the changes lasted till 24 weeks. Stem cells are proposed to act as ‘Trojan horses’ in the at-risk nervous tissue by stimulation of repair mechanisms leading to behavioral recovery after stroke.

References

1. Sethi PK. Stroke –Incidence in India and management of ischemic stroke. Neurosciences, 2002; 3: 202-206.
2. Banerjee T, Das S. Epidemiology of stroke in India. Neurology Asia. 2006; 11: 1-4.
3. Locatelli F, Bersano A, Strazzarella S, Bresolin N, Corti S. Stem cell therapy in stroke: Review. Cell Mol Life Sci. 2009; 66: 757-772.
4. Kondziolka D, Wiescher L, Meltzer C, Goldstein S. Transplantation of cultured neuronal cells for patients with stroke. Neurology. 2000; 55: 565-570.
5. Savitz SI, Dinsmore J, Wu S. Neural transplantation of fetal porcine cells in patients with basal ganglia infarcts; a preliminary safety and feasibility study. Cerebrovasc Dis. 2005; 2: 101-112.
6. Bang YO, Lee JS, Lee PH. Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol. 2005; 57: 874-881.
7. Rabinovich SS, Seledtsov VI, Banul NV. Cell therapy for brain stroke. Bull Exp Biol Med. 2005; 139: 126-134.
8. Bergado JA, Monteagudo CS, Ramirez PH, Gonzalez RM. Autologous bone marrow stem cell transplantation in stroke patients. An open study. Restorative Neurology and Neuroscience. 2009; 27: 151-161.
9. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol. 1995; 11: 35-71.
10. Domen J, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol Med Today. 1995; 5: 201-208.
11. Machalinski B, Pacczkowska E, Koziańska D, Ratjaczek MZ. Mobilization of human hematopoietic stem/progenitor-enriched CD34+ cells into peripheral blood during stress related to ischemic stroke. Folia Histochemica et Cytobiologica. 2006; 44(2): 97-101.
12. Kelly S, Bliss TM, Shah AK, Sun GH, Foo WC. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A. 2004; 101(32): 11839–11844.
13. Shintani S, Murohara T, Ikeda H, Ueno T. Augmentation of Postnatal Neovascularization With Autologous Bone Marrow Transplantation. Circulation. 2001; 103: 897-903.
14. Schwarting S, Litwak S, Hao W, Bähr M, Weise J, Neumann H. Hematopoietic Stem Cells Reduce Postischemic Inflammation and Ameliorate Ischemic Brain Injury. Stroke. 2008; 39: 2867-2875.
15. www.clinicaltrials.gov/ct2/show/NCT00761982.
16. www.clinicaltrials.gov/ct2/show/NCT00535197.
17. Brennemann M, Sharma S, Harting M, Strong R, Cox CS. Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle aged rats. Journal of Cerebral Blood flow and metabolism 2010; 30:140-149.
18. Cramer SC. Repairing the human brain after stroke: II Restorative therapies. Ann Neurol. 2008; 63: 549-560.
19. Padma MV. Restorative therapy in stroke using for stem cells. Neurology India. 2009; 4: 381-386.
20. Nudo RJ, Milliken GW, Jenkins WM, Merzenich MM. Use-dependent alterations of movement representations in primary motor cortex of adult squirrel monkeys. J Neurosci. 1996;16: 785– 807.
21. Calautti C, Baron J. Functional neuroimaging studies of motor recovery after stroke in adults: a review. Stroke. 2003; 34: 1553–1566.
22. Cho SH, Kim DG, Kim DS, Kim YH. Motor outcome according to the integrity of the corticospinal tract determined by diffusion tensor tractography in the early stage of corona radiata infarct. Neurosurgery Letters. 2007; 426: 123-127.
23. Bliss T, Guzman R, Daadi M, Steinberg GK. Cell Transplantation Therapy for Stroke. Stroke. 2007; 38:817-826.
24. Shen LH, Li Y, Chen J, Zhang J, Vanguri P, Bomeman J, Chopp M. Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodelling after stroke. Neuroscience. 2006; 137: 393–399.
25. Steinnder DA. Neural stem cells, scaffolds and chaperones. Nature. 2002; 20: 1091-1093.
26. Loewen SC, Anderson BA. Reliability of motor assessment and Barthel Index. Phy Ther. 1999; 8:1077-1081.
27. Ismail S, Bilger Y, Evren Y, Rdvani A. Brunnstrom recovery stage and motricity index for the evaluation of upper extremity in stroke: analysis for correlation and responsiveness. International Journal of Rehabilitation Research. 2009; 32(3): 228-231.
28. Oldfield RC. The assessment and analysis of handedness: the Edinburgh inventory. Neuropsychologia. 1972; 9: 97-113.
29. Gaggioli, A., Meneghini, A., Morganti, F. A Strategy for Computer-Assisted Mental Practice in Stroke Rehabilitation. Neurorehabil Neural repair. 2006; 20: 503-507.
30. Maeda F, Kleiner-Fisman G, Pascual-Leone A. Motor facilitation while observing hand actions: specificity of the effect and role of observer’s orientation. J Neurophysiol. 2002; 87: 1329–1335.
31. Steiner CM, Barber PA, Smale PR. Functional potential in chronic stroke patients depends on corticospinal tract integrity. Brain. 2007; 130: 170-180.
32. Feydy A, Carlier R, Brami AR, Bussel B, Cazalis F, Pierot L, Burnod LY, Maier MA. Longitudinal Study of Motor Recovery After Stroke Recruitment and Focusing of Brain Activation. Stroke. 2002; 33: 1610-1617.
33. Small SL, Hlustik P, Noll DC. Cerebellar hemispheric activation ipsilateral to the paretic hand correlates with functional recovery after stroke. Brain. 2002;125: 1544-1557.
34. Nair DG, Fuchs A, Burkart S, Steinberg FL, Kelso JAS. Assessing middle cerebral artery after Stroke. Brain Injury. 2005; 19(13): 1165–1176.
35. Moller M, Frandsen J. Dynamic changes in corticospinal tracts after stroke detected by fibre tracking. J Of Neurol Neurosurg Psychiatry. 2007; 78: 587-592.
36. Komitova M, Mattsson B. Enriched environment increases neural stem/ progenitor cell proliferation and neurogenesis in the stroke lesioned adult rats. Stroke. 2005; 36: 1287-1282.
37. Willing A, Lixian J, Milliken M, Poulos S. Intravenous versus intrastratial cord blood administration in a rodent model of stroke. J of Neurosci. 2009; 9: 296-307.
38. Chen J, Wang S. Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. Circ Res. 2003; 92: 1-10.
39. Shen SH, Zang Z, Lu M. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. J of Cerebral Blood flow and metabolism. 2002; 27: 6–13.
40. Jin K, Zhu Y, Sun Y. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci USA. 2002; 99: 11946–11950.
41. Wechsler L, Steindler D, Borlongan C, Chopp M, Caplan L, Hess D. Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) Bridging Basic and Clinical Science for Cellular and Neurogenic Factor Therapy in Treating Stroke. Stroke. 2009; 40: 510-515.

Abbreviations

CIS : chronic ischemic stroke
BOLD: blood oxygen level dependent
DTI : diffusion tensor imaging
CST: corticospinal tract
Rt : right
Lt: left
PT: prothrombin time
PLIC : posterior limb of internal capsule
MRC: medical research council
FA: fractional anisotropy
FN: fiber number
FL: fiber length

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Conflicts of interests

None