Intravenous Autologous Bone Marrow Mononuclear Stem Cell Therapy for Ischemic Stroke
A Multicentric, Randomized Trial

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**Background and Purpose**—Pilot studies have suggested benefit from intravenous administration of bone marrow mononuclear stem cells (BMSCs) in stroke. We explored the efficacy and safety of autologous BMSCs in subacute ischemic stroke.

**Methods**—This was a phase II, multicenter, parallel group, randomized trial with blinded outcome assessment that included 120 patients. Patients with subacute ischemic stroke were randomly assigned to the arm that received intravenous infusion of autologous BMSCs or to control arm. Coprimary clinical efficacy outcomes were Barthel Index score and modified Rankin scale at day 180. Secondary outcomes were change in infarct volume, National Institute of Health Stroke Scale (NIHSS) at day 90 and 180. Main safety outcomes were adverse events, any new area of fluorodeoxyglucose positron emission tomography uptake in any body part over 365 days.

**Results**—Fifty-eight patients received a mean of 280.75 million BMSCs at median of 18.5 days after stroke onset. There was no significant difference between BMSCs arm and control arm in the Barthel Index score (63.1 versus 63.6; \(P=0.92\)), modified Rankin scale shift analysis (\(P=0.53\) or score \(>3\) (47.5% versus 49.2%; \(P=0.85\)). NIHSS score (6.3 versus 7.0; \(P=0.53\)), change in infarct volume (−11.1 versus −7.36; \(P=0.63\)) at day 180. Adverse events were also similar in the 2 arms, and no patient showed any new area of fluorodeoxyglucose uptake.

**Conclusions**—With the methods used, results of this hitherto first randomized controlled trial indicate that intravenous infusion of BMSCs is safe, but there is no beneficial effect of treatment on stroke outcome.

**Clinical Trial Registration**—URL: http://ctri.nic.in/Clinicaltrials and http://www.clinicaltrials.gov. Unique identifiers: CTRI-ROVCTRI/2008/091/0004 and NCT0150177. (**Stroke.** 2014;45:00-00.)

**Key Words**: adult stem cells ▪ bone marrow cells ▪ cell- and tissue-based therapy ▪ cerebral infarction ▪ randomized controlled trial ▪ stem cell transplantation ▪ stroke

WHO estimates that globally ≈15 million people experience stroke annually, of which 6 million die and 4 million are left with significant disability.1 Despite advances in acute care and secondary preventive strategies, stroke remains a major burden on healthcare system worldwide. Intravenous thrombolysis is the only approved therapy for acute ischemic stroke.2 However, few stroke patients receive this therapy because of its narrow time window.3 There is a clear need for a treatment that improves neurological outcome after stroke. In experimental model of stroke, intravenous, intrastriatal, and intraarterial infusion of mononuclear stem cells from bone marrow have improved neurological outcome through reduced apoptosis, decreased peri-infarct inflammation, and angiogenesis.4–6 The transdifferentiation of transplanted BMSCs has also been reported.7 Phase I studies have shown feasibility and preliminary safety of intravenous infusion of autologous BMSCs in acute and subacute ischemic stroke,8,9 but no randomized study has been reported. Yet, there is immense interest about this therapy among health professionals, media, and public. Many private for-profit

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healthcare facilities are offering this therapy at high cost to unsuspecting and desperate patients weeks to months after stroke without any randomized evidence.\textsuperscript{10}

We conducted a randomized, multicenter study to investigate the effects of intravenous infusion of autologous BMSCs in subacute ischemic stroke (Intravenous Autologous Bone Marrow Mononuclear Cell Therapy for Ischemic Stroke [InveST]). We tested the hypothesis that in patients with subacute ischemic stroke, intravenous infusion of autologous BMSCs between 7 to 30 days after onset results in reduction of infarct volume and improvement in neurological function at day 180 of follow-up compared with those without the infusion.

**Subjects and Methods**

**Study Design and Setting**
This study was a phase II, randomized, multicenter, open-label, parallel group trial with blinded end point assessment (Figure 1). We included patients with subacute ischemic stroke from 5 postgraduate teaching hospitals fully funded by Government of India.

**Standard Protocol Approvals, Registration, and Patients Consents**
The Department of Biotechnology, Government of India, Ethics Committee and all the center ethics committees approved the protocol. The trial was registered at the Clinical Trial Registry-India (CTRI-PROVCTRI/2008/091/00046) and clinicaltrial.gov (NCT01501773). All patients or their legally authorized representatives gave written informed consent. All the procedures were followed in accordance with institutional guidelines.

**Randomization**
Patients were randomly assigned using permuted block randomization in a 1:1 ratio to receive either intravenous infusion of autologous BMSCs (BMSC arm) or to a control treatment where neither BM aspiration nor sham infusion were performed. The central data management office used a computer to generate the randomization sequence stratified by center with a varying block size of 4, 6, and 8. To ensure allocation concealment, centers had to contact the central office using e-mail, fax, or telephone. The office allotted a unique identification number to each patient and intimated allocation within 12 hours of the contact. Care providers and patients were not masked; however, the outcome (modified Rankin scale [mRS], Barthel Index [BI], and imaging) assessors were masked to treatment allocation.

**Inclusion and Exclusion Criteria**
Inclusion criteria were an age of 18 to 75 years, computed tomography, or MRI scan of the head showing relevant infarct within the middle cerebral artery or anterior cerebral artery territory and excluding a hematoma, onset of stroke between 7 and <30 days, Glasgow Coma Scale score >8, BI score of ≤50, National Institute of Health stroke scale (NIHSS) score of ≥7 and inability to walk unaided or raise upper limb by 90° and clinically stable condition for ≥48 hours. Definition of stable patients and exclusion criteria are given in Methods Ia and Ib in the online-only Data Supplement, respectively.

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**Figure 1.** Study flow diagram. BMSC indicates bone marrow mononuclear stem cells. *Includes National Institutes of Health Stroke Scale, modified Rankin scale, and Barthel Index. †This patient was followed up at day 365 through a new telephone number obtained via mail.
Study oversight and quality control measures are given in Methods II in the online-only Data Supplement.

**Intervention**

At each center, bone marrow was aspirated aseptically from the posterior iliac crest under local anesthesia (1% lidocaine). BMSCs were separated by ficoll density separation method. Quality control criteria for the release of cell product included bacterial sterility, >90% viability by trypan blue (Himedia, India) exclusion, and cell morphology by Giemsa’s staining. Immunophenotyping to count CD34+ cells was done using flow cytometry (see Methods III in the online-only Data Supplement for details). The cells suspended in phosphate-buffered saline were infused into antecephalial vein of the patients in BMSC arm.

**Monitoring for Infusion-Related Toxicity**

Besides hematologic indices, pulmonary, renal, hepatic, and renal systems were monitored. Neurological assessment was performed daily until day 7 or hospital discharge.

**Methods and Measurement**

Baseline assessment included demographic and clinical details and MRI of brain, electroencephalography, whole body 18fluorodeoxyglucose positron emission tomography (PET) scan (in 4 centers), NIHSS, and BI. All patients received conventional treatment according to current guidelines,1 but the treatment arm, in addition, received BMSCs intravenously within 48 hours of random allocation. No medical therapy was delayed or denied because of patient’s participation into the study.

**In-Hospital Assessment and Follow-Up Schedule**

Follow-up assessment and investigations were done on day 7 (including mRS), day 90, day 180, and day 365 to evaluate safety and efficacy. Follow-up MRI scans were performed at day 90 and day 180. Electroencephalographies were done at day 180 and day 365, and 18fluorodeoxyglucose-PET scan was required at 4 centers having this facility at baseline, day 180, and day 365. NIHSS and BI were administrated on day 90, day 180, and day 365 at each site. A central telephonic follow-up for all patients were done from coordinating center at day 90, day 180, and day 365 by a trained and blinded assessor (unaware of patient arm) who determined the vital status and administered the scales to surviving patients.

**Magnetic Resonance Imaging**

Standardized sequences were obtained including T1, T2 series, and a fluid-attenuated inversion recovery sequences. Volumetric analysis was performed on fluid-attenuated inversion recovery images at the central core laboratory using Analyze software (version 8.1: AnalyzeDirect, Inc, KS) to determine the volume of infracts and volume of both lateral ventricles. All images were saved in CDs and mailed to the coordinating center where they were stored, anonymized, and analyzed.

**Outcomes**

The coprimary clinical efficacy outcomes were measured by BI score and mRS score at day 180 assessed centrally by the assessor by telephone. The secondary clinical efficacy outcome was NIHSS score at day 90, day 180, and day 365. Outcome assessors were blinded to intervention and baseline scores. (A brief description of BI, NIHSS, and mRS can be found in Methods IV in the online-only Data Supplement.) The worst value on the 3 scales (mRS-6, BI-0, and NIHSS-42) was assigned to patients who died. Secondary imaging efficacy outcome was change in infarct volume between baseline and day 90 and day 180. All imaging analyses were performed centrally on deidentified data. Analysts were unaware of the treatment arms and clinical information. The safety outcomes included death, adverse events (serious and nonserious), epileptiform discharges in electroencephalography, and evidence of any new growth on PET scan at day 365.

**Sample Size**

Sample size was calculated for superiority hypothesis on the BI and mRS. Calculation based on the data in the only published controlled trial of stem cell (mesenchymal)2 yielded a sample size of 16 (standardized effect size=2), but our study used BMSCs at lower doses, and hence, we hypothesized only one third of the effect size (standardized effect size=0.68; with 90% power and α level of 5%). Calculation with standard formula3 yielded a sample size of 45 per group. Adjusting for 10% losses to follow-up and 1 interim analysis, we estimated a sample size of 120 in total. This confers 90% power to detect standard effect size of 0.6 on mRS scale score at 5% significance level.13 However, interim analysis was abandoned as recruitment had completed by the time criterion (6-month follow-up in 60 patients) for the analysis was fulfilled.

**Statistical Analysis**

Statistical analysis was based on the scores obtained by central telephonic assessment and followed intention-to-treat principle. Descriptive statistics included mean for numeric data with normal distributions, median otherwise, and proportions for categorical data. Primary efficacy analysis was shift analysis of modified Rankin scale scores using the Cochran-Mantel-Haenszel test with SAS version 9.2, unadjusted and adjusted BI score and mRS score. Secondary outcomes were analyzed using unadjusted Student t test for means and χ2 test for proportions. The analyses were repeated after adjustment for baseline scale scores, NIHSS, and infarct volume using regression methods. All data analyses (other than shift analysis) were done using SPSS v 17.0. Stratified analyses were done using RevMan v 5.1. All analyses were 2-sided, and P values <0.05 were considered statistically significant.

**Results**

Between November 2008 and June 2010, 5 centers screened 423 patients and included 120 patients meeting the eligibility criteria for the study. Of these, 60 each were assigned to the BMSC or control arm. One patient in each arm withdrew consent soon after randomization, but the one in control arm agreed for telephonic follow-up.

**Baseline Characteristics**

Baseline characteristics (Table I) were well balanced between the 2 arms, except infarct volume, which was 24.84 cm³ higher in control arm than in BMSC arm. None of the patients had received intravenous tissue-type plasminogen activator or endovascular therapy.

**Intervention**

All patients received the conventional treatment. Bone marrow aspiration was successfully completed without any adverse event in 58 patients in the BMSC arm (1 withdrew and 1 missed because of logistical difficulty). The aspiration yielded 108.9±33.9 mL of aspirate. The mean number of mononuclear cells infused was 280.75 million (SD, 162.9) containing CD34+ cells of 2.9 million (SD, 2.8). BMSCs viability was 93.2% (SD, 5.75). Median time from onset to cell infusion was 18.5 days (interquartile range [IQR], 9.2) and from randomization 1 day (IQR, 1). Median time from aspiration to infusion was 3.20 (IQR 0.4) hours. All patients were...
Follow-Up

Central telephonic follow-up was complete for 116 (96.7%) at day 90, 118 (98.3%) at day 180, 117 (97.5%) at day 365, and 118 (98.3%) at the end of the study. Five (8.4%) of 59 patients in BMSC arm and 5 (8.3%) of 60 in control arm died before day 180. Three more patients died at day 195, day 206, and day 221 in BMSC arm. Median follow-up time was 640 (IQR, 221) days in control arm and 634 (IQR, 229) days in BMSC arm.

Follow-Up Investigations

Ninety-four (84% of survivors) patients had MRI at day 90 and 87 (81% of survivors) at day 180. Of these, 84 (89%) were analyzable at day 90 and 71 (82%) at day 180. Sixty-four (59%) patients had whole body-18fluorodeoxyglucose-PET scan at day 180 and 49 (47%) at day 365. Seventy-nine (73%) patients had electroencephalography at day 180 and 78 (74%) at day 365.

Efficacy Outcomes

Coprimary Outcomes

The BI score at day 180 showed no difference between the BMSCs and control arm (Table 2). Analysis adjusted for infarct volume in cm3 ± SD (range) (n=92; BMSC arm=47, control arm=45).

Figure 2. Barthel Index (BI) score (and standard error) at days 90 and 180 shows no difference between the 2 arms.
volume, baseline NIHSS, and baseline BI did not change the results. BI score at day 90 and day 180 of the 2 arms was also similar (Figure 2). Scores of mRS in control arm versus BMSC arm at day 180 showed no difference between the BMSC and control (Table 2). Cochran-Mantel-Haenszel shift analysis of the scores did not reveal any statistically significant difference (P value=0.56 [unadjusted]; 0.53 [adjusted*]; Figure 3).

Secondary Outcomes
No significant difference in NIHSS score and change in infarct volume at day 90 and day 180 were observed between the BMSCs and control arm (Table 2). No relationship was observed between cell dose and outcomes (Results I and Table I in the online-only Data Supplement). Subgroup analysis did not show statistically significant interaction between cell dose and NIHSS and side of hemiplegia (Results II in the online-only Data Supplement).

Safety Outcomes
Kaplan-Meier survival curve was comparable between the 2 arms (Figure I in the online-only Data Supplement). The adverse events and serious adverse events were also comparable between the 2 arms (Table II in the online-only Data Supplement). Whole body 18fluorodeoxyglucose-PET did not reveal any new growth over 180 days in 64 patients and over 365 days in 56 patients (Figure II in the online-only Data Supplement). Electroencephalographies revealed epileptiform discharges in 3 patients, all in BMSCs arm but 2 before infusion and only 1 patient at both days 180 and 365 postrandomization.

Discussion
Our study is hitherto the first and the largest randomized controlled trial comparing intravenous infusion of autologous BMSCs and control in patients with subacute ischemic stroke. The findings of this open-label trial with blinded outcome assessment indicate that intravenous infusion of BMSCs at a mean of 18.5 (IQR, 9.25) days after onset of ischemic stroke is safe but does not improve neurological outcome compared with the control arm. However, this is the first study to support safety of BMSCs in a comparative study.

Our results do not confirm the reported benefit in the preclinical studies2–6 and pilot clinical studies.8,9 Several possible reasons may pertain to eligibility criteria or timing, dose, and route of cell administration. We address these one by one. Our eligibility criteria was guided by our

Table 2. Efficacy Outcomes

<table>
<thead>
<tr>
<th>Type of Outcomes</th>
<th>Outcome Measure</th>
<th>BMSC Arm</th>
<th>Control Arm</th>
<th>P Value</th>
<th>Mean Difference (95% Confidence Interval)</th>
<th>Relative Risk (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprimary efficacy outcome</td>
<td>Barthel Index score at day 180 (mean±SD) (59; 59)</td>
<td>63.1±29.6</td>
<td>63.6±29.6</td>
<td>0.92</td>
<td>−0.51 (-11.32 to 10.29)</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Shift analysis for mRS Score at day 180</td>
<td>Figure 3</td>
<td>Figure 3</td>
<td>0.56</td>
<td>0.854</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>mRS&gt;3 score at day 180 (59:59)</td>
<td>28 (47.5%)</td>
<td>29 (49.2%)</td>
<td>0.53</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Secondary efficacy outcomes</td>
<td>mRS&gt;3</td>
<td>At day 90 (57; 59)</td>
<td>31 (54.4%)</td>
<td>0.725</td>
<td>...</td>
<td>0.94 (0.68 to 1.3)</td>
</tr>
<tr>
<td></td>
<td>At day 365 (57; 60)</td>
<td>30 (52.6%)</td>
<td>31 (51.7%)</td>
<td>0.917</td>
<td>...</td>
<td>1.02 (0.72 to 1.44)</td>
</tr>
<tr>
<td></td>
<td>Mean NIHSS</td>
<td>At day 90 (49; 47)</td>
<td>7.2</td>
<td>0.76</td>
<td>0.3</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At day 180 (47; 42)</td>
<td>6.3</td>
<td>0.53</td>
<td>−0.7</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At day 365 (39; 40)</td>
<td>4.8</td>
<td>0.29</td>
<td>1.1</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Mean difference in infarct volume from 90 days to baseline (37; 35)</td>
<td>−6.65</td>
<td>−4.16</td>
<td>0.69</td>
<td>−2.48</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Mean difference in infarct volume from 180 days to baseline (32; 27)</td>
<td>−11.1</td>
<td>−7.36</td>
<td>0.63</td>
<td>−3.73</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Death at day 180 (59; 60)</td>
<td>5 (8.3%)</td>
<td>5 (8.3%)</td>
<td>1.0</td>
<td>...</td>
<td>1 (0.31 to 3.28)</td>
</tr>
</tbody>
</table>

BMSC indicates bone marrow mononuclear cell; mRS, modified Rankin scale; NA, not applicable; and NIHSS, National Institutes of Health Stroke Scale.

*Adjusted for baseline scale score for corresponding outcome, duration of week from onset to randomization, baseline NIHSS, and infarct volume.

Safety Outcomes
Kaplan-Meier survival curve was comparable between the 2 arms (Figure I in the online-only Data Supplement). The adverse events and serious adverse events were also comparable between the 2 arms (Table II in the online-only Data Supplement). Whole body 18fluorodeoxyglucose-PET did not reveal any new growth over 180 days in 64 patients and over 365 days in 56 patients (Figure II in the online-only Data Supplement). Electroencephalographies revealed epileptiform discharges in 3 patients, all in BMSCs arm but 2 before infusion and only 1 patient at both days 180 and 365 postrandomization.

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Our results do not confirm the reported benefit in the preclinical studies2–6 and pilot clinical studies.8,9 Several possible reasons may pertain to eligibility criteria or timing, dose, and route of cell administration. We address these one by one. Our eligibility criteria was guided by our

Figure 3. Distribution of modified Rankin scale scores in control arm and bone marrow mononuclear stem cells arm at day 180. Shift analysis did not show statistically significant difference (P=0.53).

Figure I. Kaplan-Meier survival curve.
pilot studies and aimed to select patients who had moderate, not mild or severe stroke. Although mild strokes have uniformly good outcome, severe stroke have generally poor outcome unlikely to respond to the intervention. Therefore, we selected moderately severe stroke. As regards timing, preclinical evidence would favor administering the intervention within the first few days, although marrow stromal cell therapy administered ≤30 days after stroke has been shown effective in rodent models, and our pilot study supported this result. However, it may be noted that cell type used in the present study was different from marrow stromal cell. We decided to administer the cells from second to fourth week for 2 reasons: First, we were aware from our pilot study that many patients with NIHSS >7 tend to deteriorate in the first week and require hemicraniectomy. Clearly the effect of hemicraniectomy, even if balanced between the groups, would have masked the effect, if any, of the cell therapy. Second, we also considered that patients are being stabilized in the first week and cytokines released from the intervention may destabilize the patients, increase serious adverse events, and prompt the Data Safety Monitoring Board to stop the trial, a threatening proposition for an emerging therapy in 2007 when the study was planned. In one study of similar patients, 2 of 10 patients required hemicraniectomy, prompting the investigators to change the eligibility criteria to avoid such patients while we designed our study to avoid the high-risk period of first week. However, late administration of cells remains a possible explanation for lack of benefit observed in this study. Still, the findings are relevant because many practitioners are offering cell therapy to patients with stroke after weeks and months of onset.

Was the dose of cells sufficient? One of our study objectives was to examine any relationship between cell dose and effect. Our patients received a median dose of 268 million cells, the estimated effective dose from our pilot study, although highest quartile had >425 million cells. We did not find any dose-response relationship, raising doubt about cause and effect relationship between BMSCs infusion and outcome, and suggesting that inadequate dose is not a likely explanation for failure to find benefit with the BMSCs. In myocardial infarction also, there has been no significant correlation between BMSCs cell dose and effect.

The issue of appropriate route of therapy is not yet settled, but >19 preclinical studies showing benefit with cell therapy have used intravenous route. Moreover, comparative studies in rodent models have shown greater or similar benefit associated with intravenous route than through intra-arterial or intracerebral route. The route of delivery does not seem to explain the lack of significant benefit in our study.

Several features of this study increase the confidence in the internal validity of findings. Experimental design, central and independent group for sequence generation, and concealed allocation from a remote site, low attrition, and blinded central outcome assessment by a trained assessor limit the risk of bias in our study, thus supporting its internal validity. However, generalizability of the findings only to conditions of the study, not for stroke within 1 week or after 1 month of onset, is one of its limitations. Future studies should focus on stroke within 1 week of stroke onset.

Limitations of this study include lack of blinding of patients or physicians, but as required by ethics committee, BM aspiration or sham infusion was not performed in our control arm. Potential bias related to patient or physician behavior and infarct volume imbalance might be expected to favor the BMSC arm, yet this arm did not perform better than the control. The study has limited power but reasonably meets the requirement for a phase II study. Although we excluded patients with Glasgow Coma Scale score ≤8, yet it is possible that our study population had too severe stroke (mean infarct volume 99.3) to benefit from cell therapy. Future studies need to take this into account.

In conclusion, under the conditions of InvеST trial, BMSC is safe but ineffective in the treatment of moderately severe subacute ischemic stroke. Until ongoing or further randomized trials show efficacy, this treatment should not be used in clinical practice, and patients should not accept such therapy without question.

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Disclosures

None.

References

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Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2014/11/06/STROKEAHA.114.007028.DC1

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SUPPLEMENTAL MATERIAL
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5. Contract Research Organisation: Manipal AcuNova, Bangalore, India
Supplemental Material

**Supplemental Methods**

**Ia  Inclusion Criteria** A patient was defined stable if he had normal body temperature and respiration, blood pressure (BP) less than mean arterial pressure of 125 mm Hg (with systolic BP>90 mm Hg), fasting venous blood sugar level less than 200 mg/dl and normal urea/electrolyte.

**Ib  Exclusion Criteria**
Exclusion criteria were 1. lacunar syndrome, 2. intubation, 3. posterior circulation stroke, 4. co-morbidity likely to limit survival to less than 3 years, 5. pre-stroke disability leading to dependence on others for activity of daily living, 6. inaccessibility for follow up, 7. allergy to local anaesthetic, 8. unwillingness to provide written informed consent, 9. symptoms suggestive of acute cardiac, 10. hepatic or renal disease, 11. pregnancy, 12. HIV positivity, or 12. participation in any other trial.

**II. Study oversight and quality control measures**

The Department of Biotechnology, Government of India, Ethics Committee and all the centre ethics committees approved the protocol. The trial was registered at the Clinical Trial Registry- India (CTRI-PROVCTR/2008/091/00046) and clinicaltrial.gov (NCT01501773). All patients or their legally authorised representatives gave written informed consent. An independent Data and Safety Monitoring Board (DSMB) was responsible for safety and data integrity, an external data management office was responsible for sequence generation, random allocation of patients, double data entry and data management. An independent CRO (contract research organisation, Manipal AcuNova, Bangalore, India) was responsible for gathering and monitoring data and site visits. All monitoring procedures were compliant with requirements of the sponsor, centre ethics committees, the ‘Ethical Guidelines for Biomedical Research on Human Subjects, Indian Council of Medical Research’ and ICH-GCP. A number of quality control measures of the study were taken. Only postgraduate teaching hospitals funded by Government of India of high repute and active in the area of stroke research were selected. Each site was provided two dedicated research personnel for this study. The study was monitored centrally by several committees: The Department of Biotechnology (DBT) ethics committee, DBT Stem Cell Task Force, Government of India, Data Safety and Monitoring Board (DSMB). DBT appointed a Contract Research Organisation (CRO) to conduct the site visits, site monitoring, for adherence to the protocol including informed consent and for verification of the entries in the case record form (CRF) with the source documents. The CRO made 10 site monitoring visits to each centre during the period of the study. Training workshops were held for standardising the bone marrow harvest, transportation of harvest; cell processing, CD34+ counting and disbursement to the treatment wards. Those assessing NIHSS were certified by web based NIH Stroke Scale (NIHSS) - English programme. Barthel index was modified to suit Indian patients and validated prior to start of study. Item 9 and 10 of NIHSS was also modified and validated in the Indian Patients. The research staffs were trained to conduct the central telephonic follow-up in administration of mRS by online mRS training programme prepared by Professor KR Lees in association with the Media Services Department of the University of Glasgow, with the assistance of an educational grant. Two independent observers assessed Barthel index and Modified Rankin Scale (mRS) to determine inter-observer variability in 20 stroke patients in a blinded manner. Excellent Inter-observer agreement was obtained (Kappa value for mRS 0.733 (P 0.001), and Cronbach’s Alpha, 0.894 for Barthel index). MRI imaging protocol was discussed and developed in a meeting of all centre radiology co-investigators and communicated to the imaging centres. Ten percent of the patients were called and assessed by the principal investigator (PI) to verify the accuracy of the telephonic assessment of endpoints.
III. Flow cytometric Analysis
Immunophenotyping was performed using flow cytometry to evaluate CD34/45+ cells. An aliquot of MNCs was suspended in phosphate buffer saline (with 1% BSA and 1% sodium azide) and centrifuged at 1200 rpm for five minutes and the pellet was resuspended in PBS. Subsequently, the cells were incubated with fluorochrome conjugated monoclonal antibodies CD34-FITC, CD45-PE (BD, Biosciences, USA) for one hour at 4°C, washed once with PBS/1% BSA, and resuspended in 300 µl wash buffer for analysis. Appropriate isotype-matched controls were used to set the instrument parameters. A total of 75,000 events were acquired using FACS LSR II flow cytometer (BD Biosciences, USA). Expression of markers was assessed by using BDFACS DIVA software (6.1.2).

IV. Detailed description of BI, NIHSS and mRS
Barthel Index (BI) Score: The Barthel Index is a score to assess the patients’ daily living activities, like feeding, grooming, bathing, dressing, bowel and bladder care, and toilet use, ambulation, transfers, and stair climbing, on a 0 to 100 scale. The patients score 0 shows complete dependence on help for their daily living activities and represents bed-ridden state and the patient who score 100 shows complete independence for daily living activities and physical functioning.

National Institute of Health Stroke Scale (NIHSS): The NIHSS is a systematic assessment tool to measure the level of impairment caused by a stroke. The NIHSS contains 15 item to assess several characteristics of brain function, including consciousness, vision, sensation, movement, speech, and language. NIHSS assess the impairment on 0 to 42 scale. Higher score represents worse and devastating and severe infarct.

Modified Rankin Scale (mRS): mRS is widely used to evaluate functional outcome measure in stroke patients ranges from 0 to 6 scale. The score 0 shows no symptom at all and the score 6 shows death.
0 No symptoms at all
1 No significant disability despite symptoms; able to carry out all usual duties and activities
2 Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3 Moderate disabilities; requiring some help, but able to walk without assistance
4 Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5 Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6 Dead

V. Bone Marrow aspiration protocol and flow cytometric analysis
After injection of local anaesthetic An 11 gauge bone marrow needle was placed into the posterior iliac crest and 5-7 cc. was aspirated with a 20 cc. syringe. Additional sites on the iliac crest were chosen and procedure repeated. Vital sign were monitored at 5 minute intervals intra-harvest and post-harvest for one hour. The procedure was stopped after maximum possible volume without significant degree of pain (up to 150 ml) of bone marrow was aspirated.

VI. MRI analysis
All MRI brain images CDs were received in coded form from all the centres and were evaluated by research worker in a blinded fashion to estimate the brain infarct volume. Analysis of brain infarct volume was done by manual outlining of region of interest in the MRI Fluid attenuated inversion recovery (FLAIR) images in Digital Imaging and Communications in Medicine (DICOM) format through Analyse 8.1 software. All FLAIR slices of MRI brain images were analyzed at the baseline, day 90 and
180. Diffusion weighted images (DWI) slices were analysed in those MRI CDs in which FLAIR images were not available. Decoding of the MRI brain images CDs was done by another research worker who was blinded to infarct volume analysis.

VII. Regression analyses
Regression analysis of dose response included only the cell therapy group with BI score as dependent variable and infarct volume and the number of BMMNCs infused, treatment arm and infarct volume as the independent variables. The analyses was repeated with dichotomized BI score (logistic regression), secondary outcomes (ordinal regression for mRS, multiple regression for NIHSS) as dependent variables and with number of CD34+ cells administered as independent variables.
Supplemental Results

I. Relationship between cell dose and outcomes

Statistical Method
Dose response relationship was examined in two ways: 1) stratified analysis with dose ($\leq$ 100 million and $> 100$ million as pre-specified; and quartiles of the dose post-hoc) as stratification variable in treatment arm and with control arms randomly divided across the strata and examining interaction between dose and effect; 2) Multivariable analysis with cell dose as independent variable and outcomes as dependent variable.

Results
Pre-specified analysis was done with BM-MNC dose < 100 million or $\geq$ 100 million did not reveal any statistically significant interaction ($P=0.61$), but the number of patients receiving < 100 million cells was small (Supplemental material Figure IIIA). To distribute the number equally a post-hoc analysis with quartiles of BM-MNCs dose was done, but this also did not show any dose-response gradient ($p$ for interaction = 0.68) (Supplemental material Figure III B). Comparison of unfavorable outcome (defined as mRS score $> 3$) stratified by cell dose (No. of MNCs infused in quartiles) did not show any dose-response gradient (Supplemental material Figure IIIC). Regression analyses also did not reveal any significant interaction between cell dose and outcome (Supplemental material Table I). Similar analysis stratified by Number of CD 34$^+$ MNCs infused for outcome ‘dependent or dead’ also did not show any dose-response gradient (Supplemental material Figure III.D). Subgroup analysis is given in. Pre-specified analyses stratified by week of stroke when cells were infused since onset for outcome ‘dependent or dead’ (defined as Barthel index $< 60$ at day 180) does not show any interaction between week of infusion and outcome (Supplemental material Figure IVA).

II. Subgroup Analyses
Test of interaction for effects across the pre-specified subgroups based on side of hemiplegia and NIHSS score ranges of 1-4, 5-8, and $>8$ were not significant, indicating that outcome did not differ across subgroups. Outcome measure stratified by baseline NIHSS motor leg and motor arm score (0-16) at day 180 and outcome measure stratified by side of infarction did not show statistically significant interaction (Supplemental Figure IVB and IVC).

III. Follow-up
The correlation coefficient was tested for the central and site follow-up (BI and mRS) at day 90, 180 and 365 for its association with clinical outcomes. The central follow-up (BI and mRS) was positively correlated with the site follow up at day 90, 180 and 365.

<table>
<thead>
<tr>
<th>Follow-up</th>
<th>Correlation coefficient between central and site follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI at day 180</td>
<td>0.82</td>
</tr>
<tr>
<td>mRS at day 180</td>
<td>0.74</td>
</tr>
</tbody>
</table>

mRS was centrally administered at the end of study in both arm, a median follow up time was 645 (IQR 224) day in control arm and 640 (IQR 229) days in BMMNC arm mean, reveals that 24% patients in control arm were death/dependent compared to 21% in BMMNC arm.
Supplemental Material

**IV. Efficacy**
We first analysed whether there was any interaction between primary outcome and study centres or week of administration of cell therapy. As there was no significant interaction in both the analyses, we proceeded to analyze all the data together.

**V. Primary outcome**
Logistic regression analysis of the cell therapy arm with dichotomized BI as dependent variable and MNC number and infarct volume as independent variables did not reveal any statistically significant dose-response gradient.

**VI. Secondary Outcome**

a) Infarct volume: There was no difference between the cell therapy arm and control arm in the reduction of infarct volume between baseline and day 90 or 180 (Table 2).

b) mRS score: Analysis using mRS score at day 90, 180 and 365, and the difference between day 180 and baseline revealed no difference between the cell therapy arm and control arm (Table 2).

c) Ordinal regression analysis adjusting for age and baseline NIHSS or infarct volume did not reveal any statistically significant association between outcome and treatment arm.

c) NIHSS: No statistical significant difference was found between BMMNCs infusion in cell therapy arm and control arm in NIHSS at day 90 and 180 (Supplementary Table IV). Multiple regression analysis adjusting for age and infarct volume did not reveal any statistically significant association between outcome and treatment arm.
## Supplemental Data Tables

### Supplemental Table I. Relationship between cell dose (independent variable) and outcomes (dependent variable)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Outcome variable</th>
<th>Type of Regression Analysis</th>
<th>No of patients</th>
<th>Coefficients* (standardized)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All patients (n=118)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>BI score at day 90</td>
<td>Linear</td>
<td>116</td>
<td>0.07</td>
<td>0.42</td>
</tr>
<tr>
<td>2</td>
<td>BI score at day 180</td>
<td>Linear</td>
<td>118</td>
<td>0.08</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>BI score (6 months-baseline)</td>
<td>Linear</td>
<td>118</td>
<td>0.08</td>
<td>0.39</td>
</tr>
<tr>
<td>4</td>
<td>BI at day 90 dichotomised (cut off &lt;60 or &gt; 60)</td>
<td>Logistic</td>
<td>116</td>
<td>0.43</td>
<td>0.33</td>
</tr>
<tr>
<td>5</td>
<td>BI at day 180 dichotomised (cut off &lt;60 or &gt; 60)</td>
<td>Logistic</td>
<td>118</td>
<td>0.56</td>
<td>0.24</td>
</tr>
<tr>
<td>6</td>
<td>mRS (3 months)</td>
<td>Ordinal</td>
<td>116</td>
<td>0.09</td>
<td>0.80</td>
</tr>
<tr>
<td>7</td>
<td>mRS (6 months)</td>
<td>Ordinal</td>
<td>118</td>
<td>0.17</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>BM-MNCs group n=58</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>BI score at day 90</td>
<td>Linear</td>
<td>56</td>
<td>0.06</td>
<td>0.65</td>
</tr>
<tr>
<td>2</td>
<td>BI score at day 180</td>
<td>Linear</td>
<td>58</td>
<td>0.14</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td>BI score (6 months-baseline)</td>
<td>Linear</td>
<td>58</td>
<td>0.14</td>
<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>BI score at day 90 (cut off &lt;60 or &gt; 60)</td>
<td>Logistic</td>
<td>57</td>
<td>0.49</td>
<td>0.35</td>
</tr>
<tr>
<td>5</td>
<td>BI score at day 180 (cut off &lt;60 or &gt; 60)</td>
<td>Logistic</td>
<td>58</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>6</td>
<td>mRS (3 months)</td>
<td>Ordinal</td>
<td>57</td>
<td>-0.08</td>
<td>0.86</td>
</tr>
<tr>
<td>7</td>
<td>mRS (6 months)</td>
<td>Ordinal</td>
<td>58</td>
<td>0.41</td>
<td>0.38</td>
</tr>
</tbody>
</table>

**Abbreviations:** BI, Barthel Index, mRS, modified Rankin scale score. *With cell dose dichotomized at median number of cells.
### Supplemental Table II. Adverse events up to 365 days of follow-up

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Time Point</th>
<th>BM-MNC arm (n=59 for 180 days and 57 for 365 days)</th>
<th>Control arm (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rise in urea &gt; 2.77 mmol/l (50 mg/dl)</td>
<td>Up to 120 hrs</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Haematological</td>
<td>Up to 120 hrs</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Hepatic</td>
<td>Up to 120 hrs</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>Serious deterioration in sensorium</td>
<td>Up to 120 hrs</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hypotension</td>
<td>Up to 120 hrs</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Oedema</td>
<td>Up to 120 hrs</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pneumonitis†</td>
<td>7th day</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Fever</td>
<td>*</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hyperglycaemia</td>
<td>*</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Hypertension†</td>
<td>*</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Septicaemia with shock†</td>
<td>*</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bilateral lower limb ischemia due to abdominal aortic occlusion†</td>
<td>*</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Frozen shoulder</td>
<td>*</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Traumatic Injury</td>
<td>*</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fracture in lower limb†</td>
<td>*</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Death †‡</td>
<td>*</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>CNS</td>
<td>7th day</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>GI</td>
<td>7th day</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Increase in standardised uptake value of breast and uterine lesion on PET scan</td>
<td>*</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>61 (33%)</td>
<td>60 (36%)</td>
</tr>
</tbody>
</table>

**Abbreviations:** BM-MNC, bone marrow mononuclear cell; Haematological: TLC > 13,500/C.mm, fall in haemoglobin; Hepatic: Increase in ALT from normal baseline value to 2X normal, Increase in AST from normal baseline value to 2X normal, Rise in bilirubin > 0.05 mmol/l (1 mg%) from baseline, Rise in alkaline phosphatase; CNS (Central nervous system) adverse events includes depression, seizure, recurrent stroke; GI adverse events includes diarrhoea, vomiting, acute tonsillitis with pharyngitis, acute gall stone pancreatitis, acute gastroenteritis with gastro intestinal bleed, acute cholecystitis with cholelithiasis

*Denotes adverse events occurring during out-patient follow-up
† Denotes serious adverse events
‡See supplemental table V for likely causes of death
Supplemental Table III: Pre-specified analysis comparing those receiving < 100 & ≥ 100 million cells with Barthel index score at day 180 (Cut off 60)

<table>
<thead>
<tr>
<th>Barthel Index score at day 180</th>
<th>Infused cell</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;100 million cells</td>
<td>≥100 million cells</td>
</tr>
<tr>
<td>&lt;60</td>
<td>2 (22.2%)</td>
<td>15 (30.6%)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>7 (77.8%)</td>
<td>34 (69.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>9 (100.0%)</td>
<td>49 (100.0%)</td>
</tr>
</tbody>
</table>

P-value: 0.61
Supplemental Material

Supplemental Table IV. Additional efficacy outcomes

<table>
<thead>
<tr>
<th>Type of outcomes</th>
<th>Outcome measure (BMMNC n; control n)</th>
<th>BMMNC arm</th>
<th>Control arm</th>
<th>P-Value</th>
<th>Relative Risk (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary efficacy outcomes</td>
<td>BI &lt; 60 at day 90 (57; 59)</td>
<td>23 (40.4%)</td>
<td>26 (44.1%)</td>
<td>0.685</td>
<td>0.92 (0.60 to 1.40)</td>
</tr>
<tr>
<td></td>
<td>at day 180 (59;59)</td>
<td>17 (28.81%)</td>
<td>22 (37.29%)</td>
<td>0.328</td>
<td>0.78 (0.47 to 1.49)</td>
</tr>
<tr>
<td></td>
<td>at day 365 (57; 60)</td>
<td>19 (33.3%)</td>
<td>22 (36.7%)</td>
<td>0.706</td>
<td>0.91 (0.55 to 1.49)</td>
</tr>
<tr>
<td>NIHSS &gt; 2</td>
<td>at day 90 (49; 47)</td>
<td>41 (83.7%)</td>
<td>42 (89.4%)</td>
<td>0.416</td>
<td>0.94 (0.8 to 1.1)</td>
</tr>
<tr>
<td></td>
<td>at day 180 (47; 42)</td>
<td>39 (83.0%)</td>
<td>36 (85.7%)</td>
<td>0.723</td>
<td>0.97 (0.81 to 1.16)</td>
</tr>
<tr>
<td></td>
<td>at day 365 (39; 40)</td>
<td>29 (74.4%)</td>
<td>33 (82.5%)</td>
<td>0.379</td>
<td>0.90 (0.71 to 1.14)</td>
</tr>
</tbody>
</table>
## Supplemental Table V. Cause of death

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Patient ID</th>
<th>Likely cause of death</th>
<th>Days after randomisation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMMNC arm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>323</td>
<td>Acute coronary syndrome</td>
<td>51 Days</td>
</tr>
<tr>
<td>2.</td>
<td>311</td>
<td>Sudden cardiac death</td>
<td>78 Days</td>
</tr>
<tr>
<td>3.</td>
<td>104</td>
<td>Pulmonary embolism</td>
<td>93 Days</td>
</tr>
<tr>
<td>4.</td>
<td>124</td>
<td>Either myocardial infarction or pulmonary embolism</td>
<td>102 Days</td>
</tr>
<tr>
<td>5.</td>
<td>506</td>
<td>Sudden cardiac death</td>
<td>109 Days</td>
</tr>
<tr>
<td>6.</td>
<td>324</td>
<td>Sudden cardiac death</td>
<td>195 Days</td>
</tr>
<tr>
<td>7.</td>
<td>405</td>
<td>Sudden cardiac death</td>
<td>206 Days</td>
</tr>
<tr>
<td>8.</td>
<td>423</td>
<td>Cardiac arrhythmia</td>
<td>221 Days</td>
</tr>
<tr>
<td><strong>Control arm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>315</td>
<td>Diabetic Keto acidosis resulting in death</td>
<td>39 Days</td>
</tr>
<tr>
<td>10.</td>
<td>421</td>
<td>Gastroenteritis with gastrointestinal bleed</td>
<td>63 Days</td>
</tr>
<tr>
<td>11.</td>
<td>103</td>
<td>Pulmonary embolism</td>
<td>63 Days</td>
</tr>
<tr>
<td>12.</td>
<td>213</td>
<td>Sudden cardiac death</td>
<td>75 Days</td>
</tr>
<tr>
<td>13.</td>
<td>223</td>
<td>Sudden cardiac death</td>
<td>137 y</td>
</tr>
</tbody>
</table>
Supplemental figures

Supplemental Figure I: Kaplan-Meier Curve showing mortality in intervention group compared with control.

Survival Functions

Log Rank p Value = 0.39
Supplemental Material

Supplemental Figure II: PET and MRI Scan

A. Baseline investigations at day 19 of stroke in a 32-yr-old male shows infarct in left MCA territory in T1 and FLAIR sequence of MR. There is increased $^{18}$FDG uptake in the area of infarct due probably to peri-infarct inflammation (imaging performed on Day 19 of stroke). On whole body $^{18}$FDG image, there is marked asymmetry in muscle uptake due to paralyzed muscles in right half of the body. B. One year follow up study after stroke shows gliosis in left MCA territory in T1 and FLAIR sequence of MR, and no $^{18}$FDG uptake. On whole body $^{18}$FDG image, there is no abnormal uptake or asymmetry noted.
Supplemental Figure III: Dose response analysis

A. Pre-specified analysis comparing outcome across cell dose (less than 100 million vs. 100 million or above). Events means Barthel Index < 60 at day 180. BM-MNC denotes bone marrow mononuclear cell

B. Post-hoc analysis comparing outcome across quartile of no. of cells infused. Events means Barthel Index < 60 at day 180. BM-MNC denotes bone marrow mononuclear cell

C. Analysis stratified according to quartiles of cell dose (No. of MNCs infused). Events denote outcome mRS score >3. Heterogeneity chi square P value of 0.28 indicates no interaction between cell dose and outcome;

D. Analysis stratified according to quartile of cell dose (No. of CD34+ infused). Events denote outcome BI < 60 at day 180. Heterogeneity chi square P value of 0.66 indicates no interaction between cell dose and outcome
Supplemental Figure IV: Analysis as per timing of cell infusion

A. Analysis stratified by week after onset of stroke when cells were infused. Events denote Barthel index (< 60) at day 180: Heterogeneity chi square P value of 0.88 indicates no interaction between week after onset and outcome.

B. Analysis stratified by baseline NIHSS motor leg and motor arm score (0-16). Events denote outcome BI < 60 at day 180. Heterogeneity chi square P value of 0.18 indicates no interaction between baseline NIHSS and outcome.

C. Outcome measure stratified by side of hemiparesis. Events denote outcome BI < 60 at day 180. Heterogeneity chi square P value of 0.73 indicates no interaction between side of hemiparesis and outcome.

Events means Barthel Index < 60 at day 180. BM-MNC denotes bone marrow mononuclear cell