

Autologous intravenous bone marrow mononuclear cell therapy for patients with subacute ischaemic stroke: a pilot study

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Background & objectives: Bone marrow mononuclear cell therapy has emerged as one of the options for the treatment of Stroke. Several preclinical studies have shown that the treatment with mononuclear cells (MNCs) can reduce the infarct size and improve the functional outcome. We evaluated the feasibility, safety and clinical outcome of administering bone marrow mononuclear cells (MNCs) intravenously to patients with subacute ischaemic stroke.

Methods: In a non-randomized phase-I clinical study, 11 consecutive, eligible and consenting patients, aged 30-70 yr with ischaemic stroke involving anterior circulation within 7 to 30 days of onset of stroke were included. Bone marrow was aspirated from iliac crest and the harvested mononuclear cells were infused into antecubital vein. Outcomes measured for safety included immediate reactions after cell infusion and evidence of tumour formation at one year in whole body PET scan. Patients were followed at week 1, 4-6, 24 and 52 to determine clinical progress using National Institute of Health Stroke Scale (NIHSS), Barthel Index (BI), modified Rankin Scale (mRS), MRI, EEG and PET. Feasibility outcomes included target-dose feasibility. Favourable clinical outcome was defined as mRS score of 2 or less or BI score of 75 to 100 at six months after stem cell therapy.

Results: Between September 2006 and April 2007, 11 patients were infused with bone-marrow mononuclear cells (mean 80 million with CD-34⁺ mean 0.92 million). Protocol was target-dose feasible in 9 patients (82%). FDG-PET scan at 24 and 52 wk in nine patients did not reveal evidence of tumour formation. Seven patients had favourable clinical outcome.

Interpretation & conclusions: Intravenous bone marrow mononuclear cell therapy appears feasible and safe in patients with subacute ischaemic stroke. Further, a randomized controlled trial to examine its efficacy is being conducted.

Key words Autologous therapy - bone marrow - ischaemic stroke - mononuclear cell

Stroke has emerged as the third commonest cause of mortality worldwide¹. Nearly 50 per cent of stroke survivors are left with disabling sequelae. Acute

ischaemic stroke is the most common type and has only tissue plasminogen activator and aspirin as therapeutic options². There is a clear need for new therapeutic

options. Several preclinical studies have shown that the treatment with mononuclear cells (MNCs) can reduce the infarct size and improve the functional outcome in animal models of stroke³⁻⁶. MNCs contain several types of stem cells including haematopoietic stem cells (HSCs)⁷ and mesenchymal stem cells (MSCs) along with the haematopoietic progenitor cells and differentiated cells. These multi-potent stem cells are chemoattracted to the lesioned area and have ability to produce and secrete various cytokines and growth factors^{8,9}. The MNCs have been well established to accelerate angiogenesis/neovascularization in a number of ischaemic diseases such as limb ischaemia¹⁰ and myocardial infarction¹¹. MNCs contribute to the proliferation of endogenous ischaemia-induced neural stem/progenitor cell after cerebral infarction through vascular niche regulation¹² and contain endothelial progenitor cells (EPCs)¹³ which contribute to revascularisation and repair of injured endothelium in stroke¹⁴. The MNCs have been shown to accelerate angiogenesis¹¹, to promote endogenous repair through proliferation of host's neural stem cells¹², to decrease neuro-degeneration⁵, to prevent apoptosis and modulate peri-infarct inflammation⁴.

To our knowledge, there has been one report of intravenous autologous therapy with bone marrow MNCs in patients with stroke¹⁵. Two pilot clinical trials of mesenchymal stem cell therapy in subacute ischaemic stroke, through intravenous route^{16,17} have shown safety, feasibility and preliminary efficacy in subacute ischaemic stroke but MSCs require cell culture for several weeks before treatment which delays the start of intervention and increases the risk of contamination. MNCs are easily harvested from the bone marrow, processed and infused within a few hours of bone marrow aspiration. Thus, MNCs offer some advantages over MSCs in the treatment of subacute phase of ischaemic stroke.

Intravenous route is easier and safer, equally or possibly more effective than intracerebral or intracarotid routes^{18,19}. Savitz *et al*²⁰ reviewed the current status of cell therapy for stroke and have justified early phase clinical trial using autologous intravenous administration of stem cells in stroke. However, with advancing age, the bone marrow is progressively replaced by fat. Therefore, a question of feasibility is whether it is possible to obtain sufficient number of mononuclear cells from bone marrow. There is also a concern whether the stem cells may cause or promote tumour formation if reinfused after processing. Therefore, the objective of this phase-I study was to

determine the feasibility, safety and clinical outcome of bone marrow mononuclear cells therapy in patients with subacute ischaemic stroke.

Material & Methods

Consecutive patients attending the neurology services of All India Institute of Medical Sciences (AIIMS), New Delhi, were considered for the study between September 2006 and April 2007 and followed up for one year. The study was approved by the Ethics Committees of AIIMS and the Department of Biotechnology (DBT), Government of India.

Inclusion criteria: Patients with stroke (defined as rapidly developing clinical symptoms and/or signs of focal loss of cerebral function, with symptoms lasting more than 24 h with no apparent cause other than that of vascular origin (modified from Hatano)²¹ were considered eligible if they fulfilled all of the following criteria: age between 30-70 yr, between 7th and 30th day after onset, computerized tomographic (CT) and/or MRI scan of the head showing no haematoma, and relevant lesions within the middle cerebral artery (MCA) and/or anterior cerebral artery (ACA) territory, Glasgow Coma Scale (GCS)²² score of above 8 (in patients with aphasia Eye and Motor score of more than 6), Barthel index (BI) score²³ of 50 or less, National Institute of Health Stroke Scale (NIHSS) score developed by National Institutes of Health of 7 or more, and inability to walk unaided or raise upper limb by 90° and clinically stable : A patient was defined as stable when he had normal respiration, was afebrile, had BP less than mean arterial pressure of 125 mmHg (but no hypotension defined as systolic BP <90 mmHg), had fasting venous blood sugar level less than 200 mg/dl and normal urea/electrolytes for previous 48 h.

Exclusion criteria: Patients meeting the above criteria were excluded from the study if they had any of the following: lacunar syndrome, intubation, co-morbidity likely to limit survival to less than three years, *e.g.* malignant diseases, hepatic or renal failure, pre-stroke disability leading to dependence on others for activities of daily living, inaccessibility for follow up, allergy to local anaesthetic, pregnancy or HIV positivity.

Patients meeting all of the inclusion and none of the exclusion criteria were approached for consent to participate in the study. Patients were included in the study only if they or their next of kin provided written informed consent/assent.

Baseline assessment: Baseline assessment included clinical and laboratory tests. Patient characteristics

and outcomes are shown in Table I. Carotid Doppler and echocardiography were done when clinically indicated as part of routine management. Flow diagram of study protocol is shown in Fig. 1. All patients had baseline MR examination within 4 wk after onset of neurological deficit with a 1.5T MR scanner (Avanto, Siemens Medical Solutions; Erlangen, Germany) using a circular polarized head coil prior to treatment. MR imaging of brain was done using standard T1, T2 FLAIR and DWI sequences. Spin echo single short echo planner sequence was used to perform DWI (TR 5000 ms, TE 96 ms, 5 mm thickness, 30% gap and 128 x 128 acquisition matrix). All the images were transferred to offline work station. Infarct volume was calculated in FLAIR Images with Analyze 7.0 developed by Biomedical Imaging Resource (BIR) at Mayo Clinic (Analyze Direct, Overland Park, USA) using Region- of -Interest module (ROI). Each patient also underwent whole body 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) with a whole body PET scanner using a Seimens Biograph LSO-PET/CT System, USA. Patients received a single dose of FDG (370 M Bq or 10 mci) intravenously, and emission scanning was performed for a total of 10 min beginning 60 min after injection.

Intervention: Bone marrow was aspirated from iliac crest under local anaesthesia by the trained neurology residents. The procedure continued till either the patient requested stopping the procedure (due to unacceptable level of discomfort/pain) or volume of 160 ml aspirate was obtained. MNCs were separated by Ficoll density centrifugation method. A small aliquot of harvested mononuclear cells was used for (i) cell count, (ii) CD-34⁺ cell count by flow cytometry, (iii) cell viability test by trypan blue dye exclusion test²⁴, and (iv) microbiological sterility testing. All the isolated mononuclear cells once received after processing (within 4 h) were immediately infused into antecubital vein over five minutes through canula in the forearm.

Outcomes: Feasibility was defined as target dose feasibility *i.e.* infusion of 40 million viable mononuclear cells intravenously within four hours of aspiration.

We assessed the safety of intravenous autologous MNCs infusion by recording the development of immediate or delayed complications recorded in a form with pre-specified list of safety parameters. The list included allergic reactions (tachycardia, fever, skin eruption, leukocytosis), local complications (haematoma, local infection at the site of bone marrow aspiration and thrombophlebitis at the site of injection), clinical features suggestive of vascular

obstruction (tachypnoea, oliguria, peripheral vascular insufficiency, worsening of stroke) and systemic complications (septicaemia, increased SGOT, SGPT or rise in blood urea, creatinine levels) and development of epileptiform discharges in brain by EEG. To evaluate tumour formation as a delayed complication, physical examination was performed including inspection of the skin and oral mucosa, and a follow up whole-body FDG-PET scan at the end of 52 wk. Favourable clinical outcome was defined as any of the following at 24 wk: NIHSS <2, A score of 0 to 2 on modified Rankin Scale (mRS)²⁴ and A BI score of 75 to 100²⁶.

The NIHSS score was recorded by a research worker certified by NIH through online training and examination²⁷. The BI, and mRS during follow up were measured by a resident blinded to baseline and previous scores on these scales. This was achieved with the use of blank data collection form at each visit.

Follow up: Patients were followed up for adverse events with clinical and laboratory evaluations. A follow up CT was done 24-48 h after the stem cell infusion to detect any haemorrhagic transformation of infarct. Patients were assessed daily during hospital stay, at wk 1 (NIHSS, BI, mRS), wk 4-6 (NIHSS, BI, mRS, EEG, MRI), wk 24 (NIHSS, BI, mRS, MRI, EEG, PET) and wk 52 (NIHSS, BI, mRS, EEG, PET). BI and mRS for one patient who did not turn up for follow up, were recorded by telephone.

Statistical analysis: Descriptive statistics were used to summarize baseline characteristics, feasibility and safety data. The data were analyzed using SPSS for Windows (Version 17.0).

Results

Eleven patients were included in this study. The number (in parentheses) completing follow up visits were: one week (all), 4-6 wk (all), 24 wk (all, one by telephone) and 52 wk (all, one by telephone). One patient was found ineligible after MRI (reported as intracerebral haemorrhage). Fig. 2 shows baseline MRI scans of three study participants.

Feasibility outcomes: Protocol was target-dose feasible in 9/11 (82%) (Table I). Mean volume of aspirate was 114.4 ml (SD 29.8, range 60 to 160 ml). The mean number of mononuclear cells obtained was 85.6 million (SD 50.5, range 21 to 188.6 million) of which CD-34 positive cells (Fig. 3) constituted an average of 1.18 per cent (SD 1.1, range .08 to 3.2) and mean per cent of viable cells was 95.8 per cent (SD 2.8, range 90 to 98). Mean number of mononuclear cells infused was

Table I. Patient's characteristics and outcome

Case number	1	2	3	4	5	6	7	8	9	10	11
Age (yr)/sex	60/F	50/M	48/M	40/M	55/F	38/M	60/F	30/F	61/M	70/F	54/M
Territory and side	Rt MCA	Lt MCA	Lt MCA	Lt MCA	Lt MCA	Lt MCA	Lt MCA	MCA and ACA	Rt MCA	MCA and ACA	Lt MCA
Previous stroke	-	-	+	-	+	-	-	+	-	-	-
GCS baseline	15	10	15	15	15	9	15	15	15	15	11
Hypertension	+	-	+	-	+	-	+	-	-	+	-
Diabetes	-	+	-	-	+	-	-	-	-	+	-
Dyslipidaemia	-	+	-	+	-	-	-	-	-	+	-
Smoker	-	-	+	-	-	+	-	-	+	-	+
Days b/w onset of stroke and infusion	29	15	18	10	26	8	9	26	11	17	18
Volume of bone marrow aspirate in ml	120	120	110	118	60	160	80	150	140	90	110
Number of cells infused (in millions)	51.30	73.32	185.75	94.66	19.44	78.42	21.17	106.92	42.92	76.32	129
Viability of cells infused	96	98	95	91	97	98	90	98	96	98	97
CD-34 ⁺ count (%)	3.34	0.26	0.66	1.33	1.03	0.59	2.33	0.53	0.44	0.08	2.38
*Favourable clinical outcome	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No
Days b/w onset of stroke and MRI	23	1	22	8	25	6	5	24	9	15	20
NIHSS (B/1 month/6 months)	7/4/1	19/16/ 15	13/12/ 10	11/7/1	12/11/6	20/16/11	12/1/0	10/8/4	5/0/0	7/0/0	19/18/-
Barthel Index (Baseline/1 month/6 months)	15/70/ 65	0/0/35	50/70/ 90	50/50/ 95	25/35/ 65	25/50/ 85	45/90/ 100	50/35/ 75	65/95/ 95	50/70/ 100	0/30/ 65
mRS (1 month/6 months)	2/3	5/4	3/1	4/1	4/3	4/3	2/0	4/3	2/1	4/1	4/3
Infarct volume (in ml) (Baseline/1 month/6 months)	93.34 /90.21 88.81	235.75/ 227.65/ -	114.49/ 112.40/ 100.49	21.26/ 17.36/ 12.64	24.54/ -/ 28.80	205.60/ 169.07/ 154.98	10.91/ 6.89/ 6.07	169.89/ -/ 141.01	10.76/ -/ 9.22	12.01/ 11.77/ 5.56	398.35/ 313.69/ -

ACA, anterior cerebral artery; MCA, middle cerebral artery; Lt, left; Rt, right; GCS, Glasgow coma scale; MRI, magnetic resonance imaging; NIHSS, National Institutes of Health Stroke Scale (score range from 0 to 42 (lower score represents better outcome and higher score represents worse outcome); BI, Barthel Index (score range from 0 to 100 (Higher score represents the better outcome and lower score represents the worse outcome); mRS, modified rankin scale score range from 0 to 5 (lower score represents better outcome and higher score represents worse outcome); (-): missing value

*Favourable clinical outcome was defined BI score of 75 to 100 or mRS score of ≤ 2

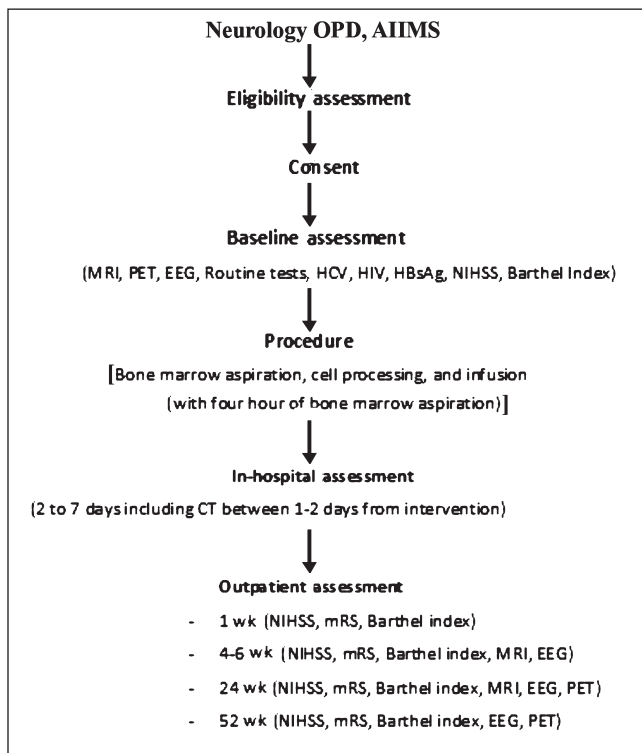


Fig. 1. Flow diagram of study protocol. MRI, magnetic resonance imaging; PET, 18F-fluorodeoxyglucose position emission tomography (FDG-PET); EEG, electroencephalography; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBsAg, hepatitis B virus antigen; NIHSS, National Institutes of Health Stroke Scale; CT, computed tomography; mRS, modified Rankin scale.

80 million (SD 48.9, range 19.4 to 185.7). All infusions were completed within four hours of bone marrow aspiration. Infusion was given in four patients during

2nd wk, three in 3rd wk, and three in 4th wk, and one at day 29 after onset of stroke.

Safety outcomes: No serious adverse event was observed during the study. The bone marrow aspiration, cell processing and cell infusion were uneventful. MRI done after 4-6 wk of the first detected a small new infarct (less than a week old) on the same side as the index one but was clinically silent. None of the patients showed deterioration in NIHSS, BI or mRS during the follow up. There was no haemorrhagic transformation of infarct in any patient. No patient developed any feature suggestive of vascular obstruction, septicemia, liver or renal dysfunction or thrombophlebitis. None of the patients' scalp EEGs showed any epileptiform discharges in the brain. Nine patients had PET scan at one year but none showed any evidence of tumour formation.

Clinical outcomes: All scales showed improvement from baseline in mean score that were statistically significant. Favourable outcome was found in 7 of 11 (64%): as per scales- BI 7 of 11 (64%), NIHSS 5 of 10 (50%) and mRS 6 of 11 (54.5%). Number of patients who achieved favourable clinical outcome with dose less than 75 million and greater than 75 million and start of stem cell therapy from the onset of stroke in weeks is represented in Table II.

Discussion

Our study suggests that use of autologous MNCs through intravenous route is safe and feasible in patients with subacute ischaemic stroke between 7 to 30 days after its onset. In the setting of subacute stroke,

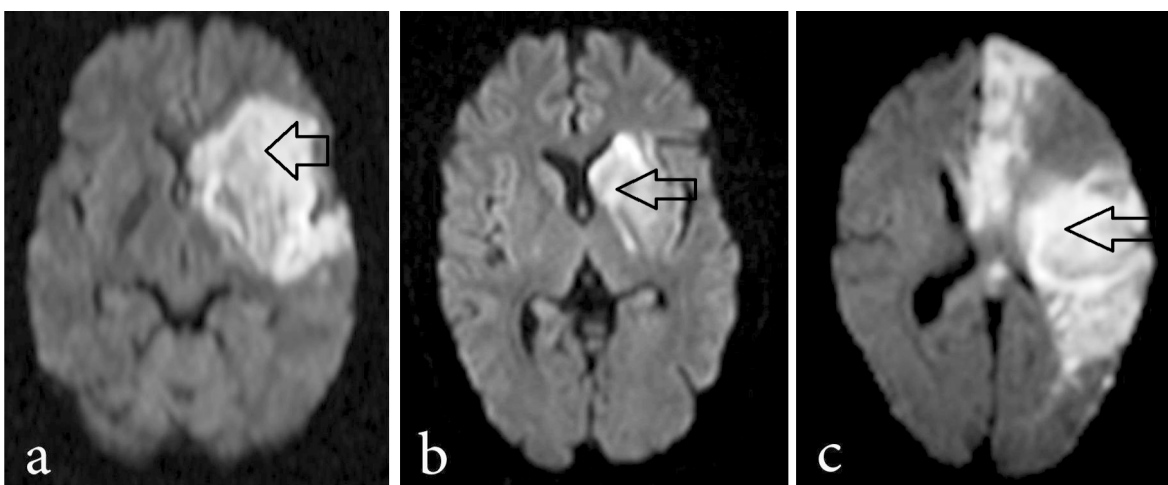


Fig. 2. Magnetic resonance imaging (diffusion weighted) showing site and size of infarct in three study patients. Arrows show location of the infarct.

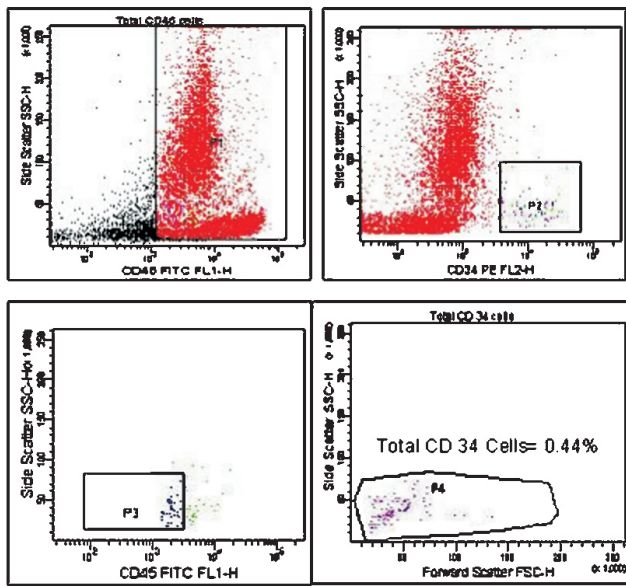


Fig. 3. Flow cytometric analysis of CD-34⁺ epitope on bone marrow derived mononuclear cells of one study participant.

a feasibility question arises: whether adequate bone marrow aspiration is possible without sedating stroke patients who are usually old in age. Sedation is avoided in patients with subacute stroke because decreased level of consciousness is an important clinical parameter to indicate deterioration in their condition that requires immediate attention. Our study shows that it is possible to successfully obtain adequate bone marrow (mean 114.36 ml), MNCs (mean 80 million) and CD-34⁺ cells (mean 0.9 million) without using sedation and from iliac crest of one side.

A safety question that often arises in the use of autologous stem cells is whether cells homing in organs

other than bone marrow, pose a risk of development of tumour. Our patients followed for one year with clinical examination and FDG-PET did not reveal any indication of such complication. Although the small number of patients with one-year follow up may not be considered definitive, the study allays fear of any major safety concern from the intervention over one year period and provides assurance to conduct further research.

Three points deserve some comments: (i) intravenous route of infusion; (ii) time window of day 7 to day 30 after onset of stroke; (iii) advantages of MNCs. Intravenous route has been the second most commonly used route in preclinical studies after intracerebral route. Two studies comparing the intravenous, intracerebral and intracarotid routes in middle cerebral artery occlusion model (MCAO) models showed that intravenous route is either superior¹⁸ or similar¹⁹ to the other routes. Meta-analysis of preclinical studies in neurological disorders favours intravenous route²⁸. Moreover, ease of administration of the cells and permissibility to use greater volume of infusate through intravenous than intracarotid route argues in favour of using intravenous route. One matter of concern with intravenous route may be the dilution of the infusate leading to small number of cells homing into the brain. Indeed, preclinical studies indicate that only 1 per cent of injected cells home into the brain¹⁸. However, even with intracarotid route, large majority of stem cells would return to venous system after first transit and undergo dilution. Considering that the major mechanism of action of MNCs seems to be through release of growth factors, angiogenesis factor and anti-apoptotic factors, it may not be important whether these home in the affected organ or other

Table II. Outcome with timing of stroke onset and bone marrow derived mononuclear stem cells infusion

Time of start of therapy from onset of stroke (wk)	No. of patients	*Favourable clinical outcome at six months	Mean difference in NIHSS from six months to baseline			Mean difference in BI from six months to baseline			Mean difference in mRS from six months to one month		
			B	6 M	Diff	B	6 M	Diff	1 M	6 M	Diff
2 + 3	8	6	12.42	5.28	-7.14	34.37	83.12	48.75	3.5	1.75	-1.75
4	3	1	9.66	3.66	-6	31.66	68.33	36.66	3.33	3	-0.33
<i>P</i> value				0.58			0.23			0.056	

Note: None of the patients had favourable clinical outcome at baseline.

B, Baseline; M, month; Diff, difference; NIHSS, National Institutes of Health Stroke Scale (score range from 0 to 42 (lower score represents better outcome and higher score represents worse outcome)); BI: Barthel Index (score range from 0 to 100 (Higher score represents the better outcome and lower score represents the worse outcome)); mRS, modified Rankin scale score range from 0 to 6 (lower score represents better outcome and higher score represents worse outcome)

*Favourable clinical outcome was defined as BI score of 75 to 100 or mRS score of <2

organs. The growth factors are probably released with change in the microenvironment of the stem cells and may enter the affected organ through blood stream and exert their effects. This is supported by a study in rat MCAo model²⁹ which suggested that homing of injected cells into brain is not a prerequisite for acute neuroprotection. Intravenous route, therefore, appears safe, effective, devoid of risks associated with catheter angiography and consistent with preclinical evidence.

We selected the time window between day 7 to 30 after onset of stroke so that at least five days are available to stabilize the patients with acute stroke and the stable state continues for at least two days before bone marrow aspiration. The upper limit of 30 days was taken because the blood brain barrier is shown to be permeable upto 30 days in most cases of moderately severe stroke³⁰ and there is experimental evidence that apoptosis continues for four to six weeks after onset of ischaemic stroke³¹. Preclinical work suggests that intravenous route maintains efficacy even when treatment is delayed for one month³². As anti-apoptosis is one of the main mechanisms of action of stem cells through intravenous route²⁹, there is potential for benefit throughout the time window used in the study. The wide window will allow us to examine this potential during various time periods in phase-2 study and if established, it increases the generalizability of the therapy.

Autologous MNCs offer certain advantages over other stem cell types: (i) being autologous substantially less risk, if any, of tumour formation compared to embryonic stem cells; (ii) no risk of rejection; and (iii) ready availability in substantial numbers compared to MSCs. Ready availability allows the early administration of MNCs after stroke onset.

Our sample size was consistent with the ongoing and completed phase-I studies registered in NIH website (www.clinicaltrials.gov) having sample size of 12 or less³³. The field of stem cell therapy is new, and phase-I studies are being undertaken on a few patients as matter of abundant caution. The results from such studies form the basis for larger phase-II studies, some of which are ongoing. Cell tracking and mechanism of action of stem cell therapy are other important issues. However, expert groups have recently noted that “there are no accepted techniques to label and monitor cells for clinical testing” and “understanding the mechanism of action is not essential before initiating clinical trials”³⁴.

The present study was small and without comparison group. Safety beyond one year was not

examined in this study. The large time window makes it difficult to ascertain average changes in functional outcomes over time. We do not know whether bone marrow volume used in our study was enough to treat stroke patients but at the current state of knowledge exact volume is unknown and hence this is a limitation common to all bone marrow autologous stem cell studies involving stem cell therapy. The preclinical study indicating efficacy at this time window period of stem cell therapy used MSCs and it is not known whether extrapolating this to MNCs is valid. Some groups used multiple antibodies to characterize different types of other cells in MNCs. One of the limitations of this study was use of only one antibody to characterize only CD-34⁺ cells.

In conclusion, our findings indicate that MNCs therapy in patients with subacute ischaemic stroke appears feasible and safe. Its efficacy needs examination in a randomized controlled trial that is already initiated (registration number CTRI -PROVCTRI/2008/091/000046)³⁵ and clinicaltrials.gov (NCT01501773).

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