Published in final edited form as:

J Neurosci Res. 2011 June ; 89(6): 833–839. doi:10.1002/jnr.22614.

Therapeutic Time Window and Dose Response of Autologous Bone Marrow Mononuclear Cells for Ischemic Stroke

Bing Yang, MD, Roger Strong, BS, Sushil Sharma, PhD, Miranda Brenneman, Kasam Mallikarjunarao, PhD, XiaoPei Xi, James C. Grotta, MD, Jaroslaw Aronowski, PhD, and Sean I. Savitz, MD

Department of Neurology, University of Texas Medical School at Houston, Houston, TX

Abstract

Although mononuclear cells (MNCs) from bone marrow are being investigated in phase I clinical trials in stroke patients, dose response, therapeutic time window and biodistribution have not been well-characterized in animal stroke models. Long Evans rats underwent common carotid artery/middle cerebral artery occlusion (CCA/MCAo) and 24 hrs later were randomized to receive saline IV or a bone marrow aspiration followed by an IV infusion of autologous separated MNCs (1 million, 10 million or 30 million cells/kg). In another experiment, rats underwent CCAo/MCAo and were randomized at 24 hrs, 72 hrs or 7 days after stroke to receive a saline injection or 10 million/kg MNCs. All animals were evaluated on the cylinder and corner tests up to 28 days. MNCs were tracked using Q-dot nanocrystals to monitor biodistribution. Animals treated with MNCs at 10 million and 30 million cells/kg at 24 hrs after stroke had significant reductions in neurological deficits and lesion size compared to saline controls or animals treated with 1 million cells/kg. There was no difference in neurological deficits in the 10 and 30 million cell/kg groups at 28 days. Animals treated with MNCs at 72 hrs but not at 7 days showed a significant reduction in neurological deficits by 28 days. Labeled MNCs were found in the brain, spleen, lung, liver, and kidney at 1 hr and exponentially decreased over the ensuing week. In conclusion, we found a maximum reduction in neurological deficits at 10 and 30 million cells/kg and a therapeutic time window up to 72 hrs after stroke.

Keywords

stem cell; stroke; bone marrow

INTRODUCTION

Cell-based therapy is actively being investigated as a new potential treatment for ischemic stroke (2009). Various types of bone marrow cells, for example, have been found to improve neurological outcome in animal stroke models. Mononuclear cells (MNCs) within the bone marrow are particularly promising because they are enriched with mesenchymal and hematopoietic stem cells, have been shown to reduce neurological deficits in animal models of stroke (Giraldi-Guimaraes et al. 2009; Iihoshi et al. 2004), they can be rapidly extracted from bone marrow and therefore permit autologous testing, they do not require growth in culture, and they have already been shown with variable results to improve outcome in patients with myocardial infarction (Lipinski et al. 2007). However, there is insufficient data on dose response and a therapeutic time window of autologous bone marrow-derived MNCs

in rodent models of stroke. In a clinically relevant model, we have recently shown that bone marrow derived MNCs can be extracted from rodents at 24 hrs after stroke and intra-arterial delivery at this time point reduces neurological deficits compared to saline-treated animals (Brenneman et al. 2010). In the current study, we show that intravenous administration of autologous MNC, extracted after stroke, also reduces neurological deficits and we characterize a dose response and therapeutic time window in this model.

METHODS AND MATERIALS

MCA occlusion

Focal ischemia of 180 min duration in male Long Evans rats that were 350 g was induced by tandem left middle cerebral artery (MCA) and left common carotid artery (CCA) occlusion (CCAO/MCAO), as described previously (Aronowski et al. 1997). For these procedures, animals were anesthetized with chloral hydrate (0.45 g/kg), and 0.25% Marcain (1ml/kg) was given locally before and after incision. In brief, the femoral artery was cannulated for blood pressure (BP) and blood gas recordings. The temperature of the temporalis muscle was monitored/controlled using a feed-forward temperature controller. The CCA was isolated through a midline incision and tagged with a suture. Two burr holes were made in the skull: a 1 × 2.5 mm rectangular burr hole to expose the left MCA, and one 1-mm-diameter burr hole to facilitate local cerebral perfusion (CP) measurement. CP in rat can be measured directly through the drill-thinned skull. A 0.005 inch diameter stainless-steel wire was placed underneath to occlude the left MCA rostral to the rhinal fissure, proximal to the major bifurcation of the MCA, and distal to the lenticulostriate arteries. The left CCA was then be occluded using Heifetz aneurysm clips. Interruption of local blood flow through the MCA was inspected under the microscope and verified with a laser Doppler flowmeter (LDF) placed over the ischemic area at 2 mm posterior and 6 mm lateral to the bregma (Aronowski et al. 1997).

Bone Marrow Harvest

We performed bone marrow harvest as previously described (Brenneman et al.) using the unimpaired limb at the designated time points after they had undergone CCAO/MCAO. The rats were anesthetized with isoflurane 1–2% in 70% O₂/30% N₂O. An incision was made through the skin to the medial aspect of the tibia. The periosteum was removed and the surgeon drilled a 1.25 × 2.5mm burr hole extending into the medullary cavity. A 20 gauge hypodermic needle was inserted into the medullary cavity and connected to a heparinized syringe. Bone marrow (1–1.5 ml) was aspirated while rotating and moving the needle back and forth. The medullary cavity was flushed with saline and the content aspirated. In the saline control group, a sham procedure was performed involving a burr hole and needle insertion of the tibia without bone marrow aspiration. The burr hole was sealed with bone wax and the skin closed with a nylon suture and 0.25% Marcain (1ml/kg) was given locally. This limited aspiration of the bone marrow provides a cell yield greater than 11 million cells per animal, does not cause impairment of the limbs and animals are able to fully participate in neurological testing (Brenneman et al. 2010).

Bone Marrow Cell Processing

As described previously², the cells from the bone marrow aspirate were triturated, centrifuged, and washed in PBS + 0.5% bovine serum albumin (BSA). Cells were then suspended in Media 199 and counted using a haemocytometer and coulter counter. The cell suspension was added on top of 20 mL Ficoll-Paque PLUS in a 50mL conical vial and then centrifuged. The MNCs were collected, washed with PBS+0.5% (BSA) and then counted. Cell viability was more than 98% by typan blue detection. Cells were then suspended in 1ml sterile, cold PBS saline at the desired concentration. The overall procedure took 2 hrs to

complete. We previously reported the immunophenotypes of MNCs from Long Evans rats using this procedure (Brenneman et al. 2010).

Delivery Route

For intravenous delivery, the femoral vein was isolated under a surgical microscope. Its distal end was ligated and a PE=10 catheter was inserted at the proximal end filled with sterile saline. One ml of MNCs or saline was infused over 5 minutes

Animal Groups

A total of 93 LE adult male rats were used in this study. In the dose-response experiments, 32 animals were subjected to CCAo/MCAo successfully and then 22 hrs later were randomly assigned to 4 groups (n=8 per group): three MNC treatment groups with different doses (1 million cells/kg, 10 million cells/kg or 30 million cells/kg) and one PBS control group. Bone marrow was harvested and MNCs were collected as described above. At 24 hrs after stroke, animals received an IV administration of MNCs. Those animals assigned to the saline-group underwent sham bone marrow procedure and received saline via intravenous injection. All animals were then serially evaluated with behavioral testing. The doses were chosen based upon calculations of MNC yield from bone marrow harvested in older patients with congestive heart failure who are currently undergoing MNC administration in a clinical trial at our center. We chose the upper limit of 30 million cells/kg based upon the cell yield of our bone marrow aspiration. In the therapeutic window experiments, 26 animals successfully underwent CCAo/MCAo and then at 72 hrs or 7 days after stroke, were randomly assigned to MNC treatment or saline control at those time points. Those animals assigned to the MNC group underwent harvest as described above and then received autologous MNCs 10 million cells/kg via an intravenous injection. This dose was chosen based on our dose response experiment. Behaviour tests were conducted up to 28 days after stroke for all groups.

Analysis of long term motor dysfunction

All sensorimotor testing was performed during the light cycle by an examiner blinded to treatment allocation. Animals were pre-tested and then tested on days 1, 7, 14, 21, 28 post-ischemia. We used a battery of sensorimotor tests sensitive to cortical damage produced by our rodent ischemia model and include the following:

Asymmetry in the use of forelimbs for postural support (cylinder test)—

Animals were placed into a plexiglas cylinder and their behavior observed for forelimb-use asymmetry during vertical movements along the wall of the cylinder. The final score = (nonimpaired forelimb movement – impaired forelimb movement)/(nonimpaired forelimb movement + impaired forelimb movement + both movement) as previously described in the rat. (Schallert et al. 2000) A total of 20 movements were recorded during the 10-min test.

Asymmetry-corner test—In the home cage, an animal was placed between two angled boards. When entering deep into the corner, both sides of the vibrissae are stimulated together. The animal then rears forward and upward, then turns back to face the open end. Twenty trials were performed for each rat and the percentage of left turns versus right turns was calculated. Only turns involving full rearing along either board were recorded.

Lesion Size Analysis

To assess whether cytoprotection is a potential mechanism associated with the behavioral recovery of MNCs, lesion size was determined in animals randomly allocated to the following treatment groups at 1 day after CCAo/MCAo: saline, 1 million MNCs/kg, 10

million/kg, or 30 million/kg (N=8 per group). At 28 days after stroke, rats were deeply anesthetized and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. Coronal 20 μ m frozen sections were cut and stained with cresyl violet to measure tissue loss of the chronic infarct using the indirect method and expressed as a percentage of the contralateral hemisphere (Yanamoto et al. 1999).

Biodistribution of Cells

Given that we found an improvement in neurological outcome and reduction in lesion size when 10 million cells/kg were administered at 24 hrs after stroke, we conducted another experiment to assess their tissue biodistribution using this dose. To track the transplanted cells in brain, Q dot nanocrystals, a red fluorescent marker (655 nm), was used. MNCs were harvested and labeled with Q-dot as described previously (Rosen et al. 2007). A commercially available kit was used to load the cells with Q-dots via a carrier protein (Qtracker 655 Cell Labeling Kit; Invitrogen). Briefly, 10 nM labeling solution was prepared according to kit directions, and approximately 0.2 ml was added to a 100-mm tissue culture dish containing MNCs. The cells were incubated at 37°C for 45–60 minutes, after which time they were washed twice with PBS. Transient CCAo/MCAo and cell administration were performed as described above (n=5 per time point). At 1 h, 3h, 6h, 12h, 24h, 72h, and 7 days after CCAo/MCAo, rats were anesthetized deeply with chloral hydrate, perfused with PBS, and perfusion-fixed with 4% paraformaldehyde in PBS. The brain, lung, spleen, liver, and kidney were removed carefully, and cut into 20- μ m-thick coronal sections. Sections were counterstained with DAPI or FITC and then visualized by fluorescence microscopy to detect Q-dot labeled cells. The number of Q-Tracker labeled cells were counted manually using Image-J (NIH) software on slides at 250 μ m intervals. Q-MNCs counted from 10 microscopic fields were averaged to represent data as Mean $\pm$ SD of 5 animals for each time point. For the brain sections, labeled cells were quantified along the borderzone area of the infarct (peri-infarct) as we have previously described (Brenneman et al. 2010).

Statistical Analysis

In all experiments, the animal surgeon and person performing the behavioral and anatomical analyses were blinded to treatment groups. The investigator was also blinded to the time points for the biodistribution studies. Data are presented as mean $\pm$ SD. For the behavioral tests,, repeated measures two-way ANOVA and the Bonferroni posttest were used for comparison among groups at different days after stroke. For the lesion size analysis, one way ANOVA was performed with post-hoc Turkey-Kramer tests. Statistical significance was set at p<0.05 level.

Results

Physiological monitoring during cell infusion

We monitored during infusion blood pressure, heart rate, arterial blood gases and temperature, all of which were unaffected by MNCs administration. There were no differences in these variables during cell or saline infusion (data not shown).

Bone Marrow Harvest and Cell Yield

We were able to conduct autologous MNC treatment in all animals randomized to receive cells. Needle aspiration from one tibia 22 h after stroke consistently without exception yielded over 10 million MNCs from one rat.

Dose response

After stroke, intravenous administration of autologous MNCs led to a dose-dependent reduction in neurological deficits on the corner and cylinder tests compared to saline-treated animals. The overall ANOVA revealed significant differences among the groups on both the cylinder and corner tests (Table). Post hoc comparisons found a difference between the groups treated with 10 million cells/kg and 1 million cells/kg but no differences between the groups treated with 10 million cells/kg versus 30 million cells/kg (Fig 1A, B).

Time window

Rats treated with autologous MNCs (10 million cells/kg) at 72 hrs showed significant reductions in neurological deficits on the corner and cylinder tests at 28 days after stroke compared to saline-treated control animals (Fig 2A, B). However, treatment at 1 week after stroke with autologous MNCs did not lead to a difference on the cylinder or corner tests. There were no significant differences on either test between MNC and saline-treated animals throughout the 28 day period (Fig 2C, D).

Infarct Lesion

At 28 days after stroke, the cerebral infarct lesion was a cavity. In animals treated with either 10 million cells/kg or 30 million cells/kg, there was a significant reduction in lesion size compared to saline-treated animals ($p < 0.05$). However, there was no difference in lesion size between animals treated with 1 million cells/kg versus saline (Figure 3).

Biodistribution

We found labeled MNCs in the peri-infarct regions as early as 1 hr after intravenous injection (Fig 4A). MNCs were also found in the spleen, lung, and liver (B–D). Figure 3 shows representative photomicrographs of the peripheral organs and brain sections demonstrating fluorescence-labeled MNCs 1 hr to 7 days after IV administration. There was an exponential decrease in the number of labeled MNCs in the peri-infarct area (Fig 4E).

Discussion

The MNC fraction of bone marrow is currently under investigation as a cellular therapy for a range of disorders. In the present study, acute intravenous administration of autologous mononuclear cells, extracted from the bone marrow 24 to 72 hrs after stroke, reduced neurological deficits in rats compared to saline-treated controls. The improvement in neurological outcome depended on the number of MNCs injected. At 4 weeks after stroke, animals that received 10 million cells/kg or more, showed significant reductions in neurological impairment. However, at the highest dose used (30 million cells/kg), there was no further improvement observed compared with 10 million cells/kg. The dose response curves indicate that extracting more than 10 million cells/kg will not lead to further benefit. In addition, 1 million cells/kg did not enhance recovery compared with saline controls. These doses were chosen based upon the expected cell yield from a limited bone marrow aspiration in patients.

In addition to exploring a dose response, we also defined a therapeutic time window of 72 hrs after stroke, using behavioral deficits as the primary outcome. When MNCs are administered at 7 days after stroke, they did not reduce neurological deficits in the CCAo/MCAo model. Therefore, our results suggest that MNCs may be effective at improving neurological outcome when given in the acute to subacute setting of stroke but are not effective at time periods beyond 3 days. However, we cannot rule out the possibility that MNCs may still have a beneficial effect between 3 and 7 days and it is possible that animals treated with MNCs at 7 days may still have achieved better recovery if we had studied their

outcome beyond 28 days. These translational studies underscore the importance of defining a dose response and therapeutic time window as recommended by the STEPS guidelines on cell-based therapies for stroke (2009).

Several previous studies have described the therapeutic effects of MNCs but differ in many important ways from the present report. Ilioshi et al (Iihoshi et al. 2004) found that MNCs (about 30 million cells/kg), extracted from the bone marrow before stroke and then administered at 12 hrs after stroke, reduced neurological deficits two weeks later. Other reports have either used syngeneic MNCs derived from donor rats (Giraldi-Guimaraes et al. 2009) or administered MNCs with an intra-carotid injection (Baker et al. 2007; Kamiya et al. 2008). We show, in this clinically relevant model, that harvesting and isolating autologous MNCs from the bone marrow 24 to 72 hrs after ischemic stroke, followed by IV re-infusion, leads to neurological improvement up to 28 days. We chose to extract, isolate and re-infuse MNCs at least 1 day after stroke which represents a potentially feasible time period when bone marrow harvest may be attempted in a clinical trial. In contrast to our study, however, syngeneic, donor MNCs were shown to attenuate neurological impairments when given up to 7 days but not at 30 days after stroke (Giraldi-Guimaraes et al. 2009). The rat stroke thermocoagulation model in that study causes a smaller infarct in the cortex (Giraldi-Guimaraes et al. 2009) compared to the CCAo/MCAo model. The disparate results raise the importance of testing potentially novel treatments in different stroke models to assess for robustness of effect and the importance of testing autologous MNCs, which may behave differently compared with syngeneic or allogeneic MNCs. Due to MHC incompatibility, administering allogeneic MNCs to patients would pose several logistical problems including the need for immunosuppression. Our methodological approach, therefore, differs from all prior published studies by attempting to simulate a potential clinical trial involving autologous MNCs extracted after stroke.

The optimal delivery route for cell-based therapies in stroke is unknown. Intravenous delivery may not be an ideal approach for some types of stem cells because IV administration leads to cell trapping in the lungs with minimal migration to the brain (Fischer et al. 2009). Within 1 hr after IV injection in this study, however, labeled MNCs did migrate to the brain into the peri-infarct area. In a prior report, intravenously injected MNCs passed through the pulmonary circulation to the arterial side, which may have resulted from their cell size being smaller than more purified stem cells (Fischer et al. 2009). The labeled cells that were found in the lungs in the present study might reflect the different types of cells within MNCs with different cell sizes. Intravenous injection also led to the distribution of MNCs to the spleen, liver and kidneys. In order to more selectively deliver cells to the brain, other studies have used an intra-carotid injection (intra-arterial) and have reported improved outcome in animal stroke models (Baker et al. 2007). While intra-arterial administration of cells carries potential risk for embolization (Walczak et al. 2008), higher mortality (Li et al. 2010), and inherent practical limitations in the acute clinical setting, future studies should be conducted to compare IV and IA delivery routes of MNCs in animal stroke models.

While IV administration delivers MNCs to the brain, labeled cells exponentially decreased and were nearly undetectable by 7 days. Labeled MNCs also decreased within the same time period in the peripheral organs as well. In our previous study, we found that MNCs within the brain after IA injection co-labeled with TUNEL, suggesting that the cells rapidly die off after systemic administration (Brenneman et al. 2010).

The mechanisms underlying the therapeutic effects of MNCs are the subject of ongoing investigation. In this report, we found that MNCs dose-dependently reduced lesion size of the chronic infarct. Ten million cells/kg led to the same degree of tissue protection compared

with 30 million cells/kg while 1 million cells/kg had no effect. This dose-response correlates with the behavioral studies and supports the hypothesis that cytoprotection is an important mechanism underlying the therapeutic effects of MNCs in this stroke model. Other studies have also shown that MNCs can reduce lesion size and protect neurons in the peri-infarct area, suggesting that the cells may release factors that promote cytoprotection (Giraldi-Guimaraes et al. 2009; Iihoshi et al. 2004). Because labeled MNCs die within a few days after injection, they likely protect peri-infarct areas and enhance recovery through paracrine mechanisms. One possible mechanism may be that MNCs reduce pro-inflammatory cytokines within the injured brain after stroke, suggesting that these cells exert immunomodulatory effects (Brenneman et al. 2010). Further studies are needed to determine if inflammatory markers such as interleukins are dose-dependently reduced by MNCs in the stroke model.

In summary, intravenous delivery of autologous MNCs at 10 and 30 million cells/kg reduced neurological deficits when given within 72 hrs after stroke. It is important, however, to note that dose responses and therapeutic time windows for cell-based therapies may also depend upon delivery routes, allogeneic, syngeneic or autologous approaches, and the type of stroke model.

Acknowledgments

This work was supported by grants from the NIH (NS064316), the American Heart Association, the Howard Hughes Medical Institute, and the Notsew Orm Sands Foundation.

References

1. 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*. 40(2):510–515. [PubMed: 19095993]
2. Aronowski J, Strong R, Grotta JC. Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. *J Cereb Blood Flow Metab*. 1997; 17(10):1048–1056. [PubMed: 9346429]
3. Baker AH, Sica V, Work LM, Williams-Ignarro S, de Nigris F, Lerman LO, Casamassimi A, Lanza A, Schiano C, Rienzo M, Ignarro LJ, Napoli C. Brain protection using autologous bone marrow cell, metalloproteinase inhibitors, and metabolic treatment in cerebral ischemia. *Proc Natl Acad Sci U S A*. 2007; 104(9):3597–3602. [PubMed: 17360688]
4. Brenneman M, Sharma S, Harting M, Strong R, Cox CS Jr, Aronowski J, Grotta JC, Savitz SI. Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J Cereb Blood Flow Metab*. 2010; 30(1):140–149. [PubMed: 19773802]
5. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS Jr. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev*. 2009; 18(5):683–692. [PubMed: 19099374]
6. Giraldi-Guimaraes A, Rezende-Lima M, Bruno FP, Mendez-Otero R. Treatment with bone marrow mononuclear cells induces functional recovery and decreases neurodegeneration after sensorimotor cortical ischemia in rats. *Brain Res*. 2009
7. Iihoshi S, Honmou O, Houkin K, Hashi K, Kocsis JD. A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. *Brain Res*. 2004; 1007(1–2):1–9. [PubMed: 15064130]
8. Kamiya N, Ueda M, Igarashi H, Nishiyama Y, Suda S, Inaba T, Katayama Y. Intra-arterial transplantation of bone marrow mononuclear cells immediately after reperfusion decreases brain injury after focal ischemia in rats. *Life Sci*. 2008; 83(11–12):433–437. [PubMed: 18727931]
9. Li L, Jiang Q, Ding G, Zhang L, Zhang ZG, Li Q, Panda S, Lu M, Ewing JR, Chopp M. Effects of administration route on migration and distribution of neural progenitor cells transplanted into rats

- with focal cerebral ischemia, an MRI study. *J Cereb Blood Flow Metab.* 2010; 30(3):653–662. [PubMed: 19888287]
10. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim HS, Kang HJ, Strauer BE, Vetrovec GW. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. *J Am Coll Cardiol.* 2007; 50(18):1761–1767. [PubMed: 17964040]
 11. Rosen AB, Kelly DJ, Schuldt AJ, Lu J, Potapova IA, Doronin SV, Robichaud KJ, Robinson RB, Rosen MR, Brink PR, Gaudette GR, Cohen IS. Finding fluorescent needles in the cardiac haystack: tracking human mesenchymal stem cells labeled with quantum dots for quantitative in vivo three-dimensional fluorescence analysis. *Stem Cells.* 2007; 25(8):2128–2138. [PubMed: 17495112]
 12. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology.* 2000; 39(5):777–787. [PubMed: 10699444]
 13. Walczak P, Zhang J, Gilad AA, Kedziorek DA, Ruiz-Cabello J, Young RG, Pittenger MF, van Zijl PC, Huang J, Bulte JW. Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke.* 2008; 39(5):1569–1574. [PubMed: 18323495]
 14. Yanamoto H, Nagata I, Nakahara I, Tohnai N, Zhang Z, Kikuchi H. Combination of intraischemic and postischemic hypothermia provides potent and persistent neuroprotection against temporary focal ischemia in rats. *Stroke.* 1999; 30(12):2720–2726. discussion 2726. [PubMed: 10583003]

Figure 1.

Long Evans rats treated with MNCs at 24 hrs after stroke have a dose-dependent improved recovery. (A) The cylinder test demonstrates preferential left forearm placement in animals that have undergone left CCAo/MCAo and IV saline injection at 24 hrs after stroke. Deficits persist up to 28 days after stroke. Animals that received 10 million cells/kg but not 1 million cell/kg showed attenuation of deficits over time ($p < 0.05$ for days 7, 14, 21, 28).

Neurological outcome at 28 days showed significant differences between 10 million cells/kg or 30 million cells/kg compared to saline but outcome did not differ between animals allocated to 10 million cells/kg and 30 million cells/kg. $N=8$ per group. (B) The corner test demonstrates preferential turning to the left in animals that have undergone left CCAo/MCAo and IV saline injection at 24 hrs after stroke. The results followed the same pattern of outcome in the different dosing groups. Animals that received 10 million/kg or 30 million cells/kg MNCs after stroke show reduction of deficits over time ($p < 0.05$ for days 14 and 28). $N=8$ per group.

Figure 2.

MNCs reduce neurological deficits when given within 72 hrs after stroke. In A and B, animals underwent CCAo/MCAo, then 72 hrs later were randomized to receive an IV injection of saline or autologous MNCs followed by neurological assessment on the cylinder (A) and corner (B) tests for 28 days after stroke (* $p < 0.05$ compared with saline controls). $N=8$ per group. In C and D, animals underwent CCAo/MCAo, then 1 week later were randomized to receive an IV injection of saline or autologous MNCs followed by neurological assessment on the cylinder and corner tests for 28 days after stroke. $N=5$ animals per group.

Figure 3. Lesion size was reduced by MNCs (either 10 or 30 million cells/kg) at 28 days after stroke. The lesion size was calculated as a percent of the contralateral cortex (* $p < 0.05$ compared with saline). Data are mean $\pm$ SD. N=8 per group.

Figure 4.

Fluorescence microscopic pictures illustrate MNCs in the brain (A), lungs (B), spleen (C), and liver (D). Green: FITC (to unspecifically label the organs); Red: Q-Tracker. Magnification: 400X. Figure E is a histogram showing a reduction in the number of labeled MNCs in the different organs. The x-axis shows time after injection in hours. N=5 per group.

Table**Overall ANOVA Results for Cylinder and Corner Tests For Different Dose Groups**

Day	Cylinder	F statistic	Corner	F statistic
1	p=.76	.39	p=0.94	0.13
3	p=0.06	7.1	p=0.32	1.2
7	p=.003	5.7 (1 vs 2, 2 vs 3)	p=0.03	3.2 (1 vs 2)
14	p=.002	5.9 (1 vs 2, 2 vs 3)	p=.006	4.9(1 vs 2, 1 vs 4)
28	p=.007	4.7 (1 vs 2, 1 vs 4)	p=.009	4.5(1 vs 2, 1 vs 4)

This table shows the various statistical comparisons of the different treatment groups over time. The F statistic is given for both cylinder and corner tests for days 1 to 28. The p-values for the F-statistic indicate that there were significant differences among the 4 treatment groups starting from day 7 to day 28 on both the cylinder and corner tests. Listed in parentheses are the specific significant differences between two individual treatment groups when comparing all 4 groups: Groups 1 = saline; 2 = 10 million cells/kg; 3 = 1 million cells/kg; 4 = 30 million cells/kg. There was a sustained, significant difference at each time point from day 7 to 28 between the saline treated group and 10 million cells/kg group on both tests. At day 30, there was also a significant difference between the saline treated group and 30 million cells/kg on both tests.