Intravenous and intra-arterial administration of bone marrow mononuclear cells after focal cerebral ischemia: Is there a difference in biodistribution and efficacy?

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Abstract Intravascular delivery of cells has been increasingly used in stroke models and clinical trials. We compared the biodistribution and therapeutic effects of bone marrow mononuclear cells (BMMCs) delivered by intra-arterial (IA) or intravenous (IV) injection after cortical ischemia. For the biodistribution analyses, BMMCs were labeled with $^{99m}$Tc. At 2 h, gamma-well counting of the brain and of the other organs evaluated did not show differences between the non-ischemic and ischemic groups or between injection routes, and the organs with the highest uptake were the liver and lungs, with low uptake in the brain. At 24 h, the liver maintained the highest activity, and a marked decrease was seen in pulmonary uptake in all groups. At this time point, although the activity in the brain remained low, the lesioned hemisphere showed greater homing than the contralateral hemisphere, for both the IV and IA ischemic groups. Histological analysis by CellTrace labeling indicated similar homing between both routes in the peri-infarct region 24 h after
transplantation and functional recovery was observed in both groups up to 11 weeks after the lesion. In conclusion, transplantation of BMMCs by IA or IV routes may lead to similar brain homing and therapeutic efficacy after experimental stroke. © 2012 Elsevier B.V. All rights reserved.

Introduction

Stroke remains a leading cause of adult disability worldwide. In Brazil it is the leading cause of death, with a mortality rate of approximately 51.6 deaths per 100,000 inhabitants (Garcia et al., 2009) and shows the highest case-fatality rate in Latin America (Lotufo and Bensenor, 2009). Recently, cell-based therapies have emerged as a promising tool for the treatment of stroke (Baker et al., 2007; Hicks et al., 2009; Lindvall and Kokaia, 2010; Ohtaki et al., 2008; Shimada and Spees, 2011) and different cell types and routes have been used in these studies. In order to be able to translate these results to a clinical setting, more information is required regarding the safety and efficacy of the different cell types, doses, and routes of administration. Therapy with BMMCs has led to functional improvement in animal models of focal cerebral ischemia when the cells were transplanted either intravenously or intra-arterially (Brenneman et al., 2010; de Vasconcelos Dos Santos et al., 2010; Giraldi-Guimaraes et al., 2009; Iihoshi et al., 2004; Nakano-Doi et al., 2010). Independent of the delivery route, several studies using labeled cells from different tissues and species donors suggest the existence of a high rate of cell entrapment in peripheral organs (Detante et al., 2009; Fischer et al., 2009; Gao et al., 2001). Moreover, the number of injected cells reaching the brain parenchyma seems to be small (Detante et al., 2009; Lappalainen et al., 2008). Despite the small number of cells found in the brain tissue, functional effects have been observed in the cell-treated animals, suggesting that peripheral mechanisms may play a systemic role in cell therapy, i.e., the cells do not necessarily need to reach the central nervous system in order to trigger their therapeutic effects (Borlongan et al., 2004; Mendez-Otero et al., 2007). In this respect, a careful evaluation of the biodistribution of the transplanted cells and a correlation with the functional efficacy may provide a better understanding of the mechanisms involved in the therapeutic effects observed. Nuclear Medicine techniques provide valuable means for monitoring cell therapies in vivo, with high image quality. In addition, these techniques also allow us to estimate the number of cells in different organs and tissues by counting the activity in the isolated organs (Detante et al., 2009; Lassance et al., 2009; Quintanilha et al., 2008). Our working hypothesis is that the effect of BMMCs may not depend on the intravascular modality of administration. Therefore, the present study examined the therapeutic effect after IV and IA administrations of BMMCs. We also investigated the distribution of these cells, to determine whether there are differences in effectiveness between these routes of administration.

Results

Whole-body nuclear imaging

Whole-body scintigraphies performed at 2 h after transplantation indicated high activity in the head after IA injection, both in ischemic (Figure 1) and non-ischemic groups (data not shown). After IV injection, no marked uptake was seen in the head in ischemic (Figure 1) or non-ischemic groups (data not shown). High activity in the lungs, liver and kidneys was observed in all groups in this time frame. Scintigraphy of the isolated brain, liver, spleen, kidneys, and lungs and of the head without the brain showed a high uptake in the head of IA animals and low uptake in the brain (Supplemental Figure 1).

After 24 h, the activity decreased significantly due to the 6-h half-life of $^{99m}$Tc, and the image resolution was poor. Nevertheless, a clear reduction could be observed in the lung uptake, and activity was limited mainly to the liver and kidneys in all groups. Also, the activity in the head of intra-arterial groups observed at 2 h was no longer present (Figure 1).

Biodistribution by isolated organ gamma-well counting

At 2 h after $^{99m}$Tc-BMMC transplantation, no difference in the gamma-well counting was observed between non-ischemic and ischemic groups or between intra-arterial and intravenous injections in the different organs evaluated (Figure 2). The total activity in the organs evaluated was approximately 55.2% of the injected dose and did not differ among groups. Moreover, no difference in the activity was observed between the right and left hemispheres at 2 h, in both ischemic and non-ischemic groups (Figure 2). At 24 h, only 12.3% of the initial activity injected remained in the organs, and the only organ that showed a difference in distribution among groups was the brain. At this time point, the left hemisphere showed higher activity than the right hemisphere, for both the intravenous $(5.9 \pm 3.9 \times 10^{-3} \% \text{ID/g}}$ versus $1.8 \pm 1.2 \times 10^{-3} \% \text{ID/g}; p=0.0065$; paired $t$-test; Figure 3) and intra-arterial groups $(4.2 \pm 3.3 \times 10^{-3} \% \text{ID/g}}$ versus $1.6 \pm 0.8 \times 10^{-3} \% \text{ID/g}; p=0.0472; Figure 3). In the non-ischemic animals, no difference in activity between hemispheres was seen (Figure 3). At 2 h, the organ with the highest percentage of uptake in all groups was the liver (32.5±4.5%), followed by the lungs (9.6±4.8%), kidneys (8.7±4.2%) and spleen (3.3±1.5%). With respect to the percentage of uptake per gram of tissue, the lungs showed the highest activity (5.7±3.4% ID/g), followed by the kidneys (3.6±2.4% ID/g), spleen (3.4±1.6% ID/g) and liver (2.9±0.6% ID/g). At 24 h, the organ with the highest percentage of uptake was the liver (8.1±1.7%), followed by the kidneys (3.0±1.3%) and spleen (0.86±0.2%). With respect to the percentage of uptake per gram of tissue, the kidneys showed the highest activity (1.3±0.7% ID/g), followed by the spleen (1.0±0.2% ID/g) and liver (0.6±0.1% ID/g). The high activity in the lungs observed in vivo images and quantified in gamma-well counting indicated an initial trapping at 2 h and a delayed redistribution at 24 h, with a decrease of 97% of its initial uptake, the greatest among the organs studied.

BMMCs in the periphery of the ischemic lesion

To evaluate whether the uptake in the brain in the gamma-well counting was indeed correlated with the presence of
transplanted cells in the ischemic hemisphere, we labeled the BMMCs with CellTrace and performed a histological analysis. A small number of labeled BMMCs were found only in the infarct and peri-infarct regions 24 h after transplantation, and no cells were found in the contralateral hemisphere. Figure 4A shows a photomicrograph demonstrating fluorescence-labeled BMMCs that migrated to the cerebral peri-infarct parenchyma. Cells could not be quantified in the lesion core due to the formation of the cavitation. There was no difference in the number of CellTrace-labeled BMMCs in ischemic animals 24 h after IV and IA transplantations (Figure 4B).

**BMMCs promote therapeutic effects independent of the administration route**

We have previously demonstrated functional recovery after intravenous transplantation of BMMCs in an animal model of cortical ischemia (de Vasconcelos Dos Santos et al., 2010; Giraldi-Guimaraes et al., 2009). In the present study, we investigated whether there are differences in therapeutic efficacy between intra-arterial and intravenous administration routes. To answer this question, we used the cylinder test to assess functional recovery (Schallert, 2006; Schallert et al., 2001). This test is a sensitive tool to

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**Figure 1** Representative whole-body images demonstrating the biodistribution of BMMCs labeled with $^{99m}$Tc, 2 h and 24 h (n=6 for each group) after intravenous or intra-arterial injection in ischemic rats. The intra-arterial group had significant activity in the head at 2 h. The remaining activity could be seen in the lungs, liver, kidneys and bladder in all groups at 2 h, and in the liver and kidneys at 24 h.

**Figure 2** Distribution of BMMCs labeled with $^{99m}$Tc 2 h (A) and 24 h (B) after intravenous or intra-arterial injection in non-ischemic (n=4) and ischemic rats (n=6) by gamma-well counting. Tracer activity is expressed as the percentage of injected dose per organ (%dose/organ; A and B) and dose per gram of organ (%ID/g; C and D).
evaluate sensorimotor function in the thermocoagulation model of ischemia (de Vasconcelos Dos Santos et al., 2010; Giraldi-Guimaraes et al., 2009). We observed significant functional improvement after administration of BMMCs in both groups, independent of the route of administration (Figure 5). The pre-ischemia score showed no asymmetry before lesion induction, as expected, whereas, 1 day after lesion, all ischemic animals showed clear asymmetry in the use of the forelimbs, preferentially using the limb ipsilateral to the cortical lesion, and were therefore not affected by the procedure (Figure 5). BMMCs were administered 1 PID, and from the 6th day after injection, evident recovery of sensorimotor function was observed in both the IA and IV groups. The repeated measures two-way ANOVA comparing the treated groups with the saline group revealed significant effects of time ($F = 34.91; p < 0.0001$), treatment ($F = 48.08; p < 0.0001$), and interaction between time and treatment ($F = 6780; p < 0.0001$). The analysis with two-way ANOVA and Bonferroni post-test showed significant differences in the symmetry score between the treated groups (IA and IV) and the saline group at each PID starting 7 days after ischemia (Supplemental Figure 2).

Discussion
An ever-increasing number of reports have demonstrated that in animal subjects, BMMCs improve functional outcome after ischemia (Baker et al., 2007; Brenneman et al., 2010; Giraldi-Guimaraes et al., 2009; Iihoshi et al., 2004; Kamiya et al., 2008). More recently, these cells have also been evaluated for safety and feasibility in clinical trials in patients with stroke (Battistella et al., 2011; Mendez-Otero et al., 2007; Mendonça et al., 2006). In these studies, the cells were delivered intra-arterially by a catheter in the middle cerebral artery, and the results showed that no patients exhibited adverse effects after transplantation, or worsening in neurological performance. However, IA injection requires a cerebral angiography, an invasive procedure that involves infrequent but significant risks, including new strokes. A retrospective study reviewing cerebral angiographies in nearly 20,000 patients has indicated that the risk of complications due to the procedure is approximately 2% and that the risk of a new stroke is approximately 0.14% (Kaufmann et al., 2007). Besides the intrinsic risks of a cerebral angiography, it is possible that the injection of cells may create risks such as embolisms. A preclinical study using MSCs found that despite the benefits of intra-arterial delivery of stem cells to the ischemic brain, there was a clear risk of vascular occlusion and also a large increase in mortality (67% for IA transplantation compared to 7% in non-transplanted animals) (Walczak et al., 2008). Similarly, Li et al. observed a high mortality rate (41%) after IA administration, compared with intra-cerebral (17%) and IV (8%), indicating that IV administration may be safer than the other routes (Li et al., 2010). On the other hand, a study that performed IA administration of BMMCs did not show alteration in the cerebral perfusion, suggesting that this effect may be related to the type of transplanted cells (Brenneman et al., 2010). Even if the IA route is shown to provide greater homing in the lesion, more studies are required to elucidate if the IA route is more effective than the IV and of comparable safety. This is especially relevant when considering that different groups have suggested that the effect of cell therapy in stroke may not be directly related to the presence of cells in the brain, since it is possible to observe functional recovery without the presence of cells in the cerebral parenchyma (Borlongan et al., 2004) or with few cells, as shown by studies involving different types of cell therapies after IA or IV administration (Bacigaluppi et al., 2009; Brenneman et al.,

Figure 3  Brain distribution of BMMCs labeled with $^{99mTc}$, 2 h (A) and 24 h (B) after intravenous or intra-arterial injection in non-ischemic (n=4) and ischemic (n=6) rats by gamma-well counting. Tracer activity is expressed as the percentage of injected dose per gram of organ (%ID/g) * p<0.05 paired t-test.
Because of the possible involvement of other organs, and mainly to compare the distribution between the IA and IV routes, we labeled the BMMCs with $^{99m}$Tc. Intense uptake in the head was detected by the whole-body scintigraphies, showing that transplanted cells reached some tissue either inside the brain or in other surrounding structures. Surprisingly, scintigraphy analysis of the head and other organs after removal of the brain, revealed that the high uptake continued in the head 2 h after the transplant. Analysis by gamma-well counting in the isolated organs confirmed that the high activity in the head observed in the whole-body images did not occur in the brain of the animal that received IA injection. Histological analysis after CellTrace labeling also indicated a small number of BMMCs in the peri-infarct, as shown in the graph (B). Nuclei were visualized with Sytox green (green). Scale bar: 20 μm.

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Figure 4 BMMCs in the periphery of the injury. Fluorescence photomicrography of the lesioned ipsilateral hemisphere 24 h after transplantation of CellTrace-labeled BMMCs (red; A). Both IV and IA (n = 4 for each group) treated animals showed similar numbers of BMMCs in the peri-infarct, as shown in the graph (B). Nuclei were visualized with Sytox green (green). Scale bar: 20 μm.

Figure 5 Intravenous and intra-arterial administrations of BMMCs showed a similar level of functional recovery in the cylinder test. Analysis of the sensorimotor function in the cylinder test showed a significant recovery of the impaired forelimb in both IA (n = 5) and IV (n = 8) groups compared with the saline group (n = 5 IA, n = 5 IV). This effect was maintained for 11 weeks, corresponding to the period studied, and no significant difference was observed between the IA and IV groups. Data shown in the graph are means ± S.E.M. (*) and (**) represent comparisons of the IV and IA groups respectively, with the saline groups. *** = p < 0.001, ## = p < 0.01; Bonferroni post-test.

animals had greater activity than non-ischemic animals in the lesioned (left) hemisphere compared to the non-lesioned (right) hemisphere at 24 h, and this occurred after both IV and IA injections. These two pieces of data together suggest that a significant part of the transplanted cells may have homed to a tissue outside the brain but still within the head. A potential explanation is that since the injection was made in the common carotid artery, a significant amount of cells could have been distributed along the tissue irrigated by the external carotid artery, such as the nasopharynx region. Importantly, 24 h after the transplant, the strong signal was no longer present in the head of the IA group, indicating that the homing to this region was transitory. The amount of cells that migrated to the brain at 2 h and 24 h was very small, which is in accordance with previous studies (Detante et al., 2009; Gao et al., 2001) and suggests that cells may not need to remain in the brain to generate functional improvement. In a previous study, we demonstrated a therapeutic window for IV administration of BMMCs up to 7 days after injury, with a better outcome when the cells were injected 1 day post-ischemia (de Vasconcelos Dos Santos et al., 2010). In that report, the treated animals reached a score of approximately 80% functional recovery. In an attempt to investigate whether the IA delivery could overcome this effect, we evaluated this procedure with IV administration, using the optimum time window and dose identified in the previous studies. Our results showed that there was no significant difference in the efficacy of the therapy between the animal groups that received IA and IV, and both showed functional improvement compared with the control (saline-treated) group. Different results were reported by Kamiya et al. (2008). In their study, IA administration of BMMCs led to a decrease in total infarct volume and improvement in motor function, as assessed with a rotarod test, when compared to the vehicle group, and this
effect was not seen in the IV group. The data in Kamiya and colleagues’ report were generated from the analysis of animals in which transient ischemia was performed by middle cerebral artery occlusion (MCAO), differently from our study, where the lesion was restricted to the cerebral cortex by thermocoagulation (TCL), which causes a permanent ischemia. Additionally, the number of injected cells used in our model was 3 times higher than the number used by Kamiya and colleagues, and the injection was performed 24 h after the ischemia, instead of immediately after the transient MCAO. Furthermore, Kamiya’s group conducted the histological analysis at an earlier time point, 1 h after the re-perfusion, and initially entrapped cells may no longer persist at subsequent time points. These differences may be responsible for the conflict in findings between our reports.

Conclusions

To our knowledge, this is the first study to compare IA and IV routes of administration of BMMCs for the therapy of cortical ischemia, produced in a model of thermocoagulation, in terms of biodistribution and efficacy. Twenty-four hours after cell transplantation, homing in the brain seems to be comparable between both routes in ischemic animals as analyzed by 99mTc and CellTrace labeling, and greater than that of non-ischemic animals. Moreover, the functional studies indicate a similar recovery of the sensorimotor function, as evaluated by the cylinder test, up to 11 weeks post-ischemia after IV or IA BMMC therapy.

Material and methods

Animals

Experiments were performed in adult male Wistar rats (3 to 5 months old) weighing 250 to 450 g. Our experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23), and were approved by the Institutional Committee for the Use of Experimental Animals (IBCCF 076).

Surgery

The ischemic lesion was induced by thermocoagulation of the blood in the pial blood vessels of sensorimotor cortices, as described previously (Szele et al., 1995). Briefly, the animals were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and placed in a stereotactic frame. The skull was surgically exposed and a craniotomy was performed, exposing the left frontoparietal cortex (+2 to 6 mm A.P. from the Bregma). The blood in the pial vessels was thermocoagulated transdurally by bringing a hot probe close to the dura mater. The color of the blood vessels is normally light red, and we considered the blood completely thermocoagulated after it turned to dark red. Care was taken to avoid touching and tearing the dura mater. After the procedure, the skin was sutured and the animals were kept warm under a heat lamp and returned to the colony room after they recovered from the anesthesia.

Obtaining BMMCs

BMMCs were isolated from the tibias and femurs of normal syngeneic donor rats. Briefly, bone marrow was aspirated from the bones and dissociated with serum-free DMEM-F12 (GIBCO BRL, Grand Island, NY, USA) and collected in sterile tubes. Bone-marrow cells were then mechanically dissociated, centrifuged for 5 min (250 x g), and resuspended in 4 ml of DMEM-F12. This volume was gently added over 4 ml of Histo-Topaque 1083 (Sigma-Aldrich, São Paulo, Brazil) and centrifuged for 30 min (400 x g). The cells were collected from the mononuclear cell layer and washed with phosphate-buffered saline (PBS), pH 7.4 in three consecutive series of centrifugations at 250 x g (5 min). Following a final centrifugation, approximately 3 x 10^7 BMMCs were suspended in 500 µl of saline and either injected immediately in the animals submitted to the behavior test, or labeled for distribution analysis.

BMMC labeling

BMMCs were labeled with 99mTc following protocols described previously (Barbosa da Fonseca et al., 2010; Battistella et al., 2011; Carvalho et al., 2008; Lassance et al., 2009; Quintanilha et al., 2008). Briefly, 500 µl of sterile SnCl2 solution was added to the cell suspension in phosphate-buffered saline (PBS), pH 7.4 in three consecutive series of centrifugations at 250 x g (5 min). Then, 5 mCi 99mTc was added and the incubation continued for another 10 min. After centrifugation (500 x g for 5 min), the supernatant was removed and the cells were washed three times with PBS. Viability of the labeled cells was assessed by the trypan blue exclusion test, and was estimated to be greater than 93% in all cases. Labeling efficiency (%) was calculated by the activity in the pellet divided by the sum of the radioactive in the pellet plus supernatant, and was estimated to be greater than 90% in all cases. Approximately 3 x 10^7 99mTc-BMMCs were injected in ischemic rats (1 day after ischemia) and non-ischemic rats through the jugular vein (IV) and common carotid artery (IA), immediately after labeling. For histochemical analyses, the BMMCs were incubated with CellTrace™ Far Red DDAO-SE (Invitrogen) diluted in DMEM (1:500) for 45 min at 37 °C in a 5% CO2 incubator. The same amount of cells (3 x 10^7 99mTc-BMMCs) was injected in ischemic animals by IV (n=4) and IA (n=4) routes after CellTrace labeling. Twenty-four hours after transplantation, the animals were euthanized and fixed with 4% paraformaldehyde. Three sequential coronal slices were obtained from three distinct antero-posterior regions (2.2, 1.0, and 0.4 mm from the Bregma). CellTrace-positive cells were counted in the photomicrographs obtained from the entire periphery of the lesion, by confocal microscopy. The photomicrographs were taken using a 20x objective, averaged across the 6–7 photomicrographs in each of the slices. Counts were made in a double-blind manner, and the statistical analysis was done using GraphPad Prism 5.02 (1992–2004 GraphPad Software, Inc.), using a paired t-test.
Biodistribution analysis

Whole-body nuclear imaging was performed for qualitative biodistribution in a GE Millennium Gamma Camera (General Electric Medical Systems, Milwaukee, Wisconsin, USA) equipped with a high-resolution collimator. A 15% energy window centered on the 140 keV photo peak of $^{99m}$Tc was used. At 2 h and 24 h after transplantation, animals were divided into two major experimental groups, ischemic ($n=6$) and non-ischemic ($n=4$), receiving injection of $^{99m}$Tc-BMMCs by IA or IV route, in a total of 8 groups (supplemental Figure 2). For quantitative biodistribution analysis, immediately after whole-body nuclear imaging, the rats were euthanized and the left and right cerebral hemispheres, heart, lungs, liver, kidneys, spleen and stomach were removed and weighed. A scintigraphy of the isolated brain, heart, liver, spleen, kidneys, lungs, and stomach and of the head without the brain was also performed in the ischemic animals 2 h after cell injection. The total radioactivity injected in each animal and the remaining radioactivity in each organ were measured in a gamma counter (Cobra II Auto-Gamma, Packard, USA). The percentage of the dose per organ [% dose/organ: each organ counts/total injected dose (ID)] per gram of tissue [%ID/g:% dose/organ/mass (g)] were determined for each sample.

Behavioral testing

Functional recovery was evaluated using the cylinder test (Schallert, 2006; Schallert et al., 2001), which allows measurement of forelimb use asymmetry. The analyses were performed by blinded investigators to avoid bias. Animals of all experimental groups were subjected to one trial on the pre-ischemic day and then weekly until 77 post-ischemic days (PID). The trial consisted of placing the animal inside a glass cylinder (20 cm in diameter and 30 cm in height). To prevent habituation to the cylinder, the number of movements recorded in any one trial was limited to 20. The occurrence of sole use of the ipsilateral (to the lesion) or contralateral forelimb or the simultaneous use of both forelimbs were counted. For each animal on each of the examined PIDs, the percentage relative to the total number of uses (ipsilateral + contralateral + simultaneous) was calculated for the ipsilateral (unimpaired) uses and for the contralateral (impaired) uses. Then, one asymmetry score for each animal was calculated on each PID by the following formula: asymmetry score = (% of ipsilateral uses) - (% of contralateral uses). Following this, the asymmetry score was converted to the symmetry score (100 – asymmetry score). The animals submitted to functional evaluation were tested before the ischemia, 1 PID, when they received the transplant, and weekly until 11 weeks.

Statistical analysis

For statistical analysis of the behavioral tests, repeated measures two-way ANOVA was used for comparison among groups. Because a significant interaction was observed in the cylinder test, a Bonferroni post-test was performed for each PID. The level of significance was always set at $p<0.05$. The biodistribution was analyzed using the paired t-test.

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References


