Bone-marrow-derived mesenchymal stem cells attenuate cognitive deficits in an endothelin-1 rat model of stroke

S.A. Lowrance b, K.D. Finke b, A. Crane b, J. Matyas b, J.J. Matchynski b,∗, J.J. Matchynski b,∗, T. Thibo b,∗, T. Reinke b,∗, J. Kippe b,∗, C. Hoffmann b, M. Sandstrom b, J. Rossignol b,∗, and G.L. Dunbar b,∗

Abstract

Purpose: Stroke is the third leading cause of death and permanent disability in the United States, often producing long-term cognitive impairments, which are not easily recapitulated in animal models. The goals of this study were to assess whether: (1) the endothelin-1 (ET-1) model of chronic stroke produced discernable cognitive deficits; (2) a spatial operant reversal task (SORT) would accurately measure memory deficits in this model; and (3) bone-marrow-derived mesenchymal stem cells (BMMSCs) could reduce any observed deficits.

Methods: Rats were given unilateral intracerebral injections of vehicle or ET-1, a stroke-inducing agent, near the middle cerebral artery. Seven days later, they were given intrastriatal injections of BMMSCs or vehicle, near the ischemic penumbra. The cognitive abilities of the rats were assessed on a novel SORT, which was designed to efficiently distinguish cognitive deficits from potential motoric confounds.

Results: Rats given ET-1 had significantly more cognitive errors at six weeks post-stroke on the SORT, and that these deficits were attenuated by BMMSC transplants.

Conclusions: These findings indicate that: (1) the ET-1 model produces chronic cognitive deficits; (2) the SORT efficiently measures cognitive deficits that are not confounded by motoric impairments; and (3) BMMSCs may be a viable treatment for stroke-induced cognitive dysfunction.

Keywords: Endothelin-1, mesenchymal stem cells, stroke, operant conditioning, cognitive dysfunction

1. Introduction

Stroke is the third leading cause of death in the United States, with 795,000 new or recurring strokes reported annually, making it one of the leading causes of permanent disability (Roger et al., 2012). Ischemic stroke occurs when one or more blood vessels become occluded, usually by a blood clot, leading to a lack of oxygen and nutrients to surrounding cells. This
blockage can lead to a cascade of post-ischemic damage (Iadecola & Anrather, 2011), which can be permanently debilitating or fatal.

Currently, acute stroke interventions focus on removing the blockage by injection of tissue plasminogen activators, which can break up the clot, restoring blood flow (NINDS, 1995), mechanical thrombectomy (Baker et al., 2011), or stenting (Brekenfeld et al., 2009), but these strategies are available to only a small group of patients (approximately 3–8% of stroke patients) due to limited therapeutic window, restrictions on age, and risk of cerebral hemorrhage (Reeves et al., 2005). Although these therapies are extremely valuable in the clinic, once revascularization has occurred, patients still face long-term recovery, in which cognitive (Desmond et al., 1996; Patel et al., 2002) and motoric impairment (Wade & Hewer, 1987) are common, underlying the need for restorative therapies.

Stem cells, particularly bone-marrow-derived mesenchymal stem cells (BMMSCs), have shown promise as a potential treatment option in patients with stroke (Honmou et al., 2011; Lee et al., 2010). Although BMMSCs are not pluripotent, which may limit their regenerative capacity, they have been shown to create a more suitable microenvironment via the release of neurotrophic factors, such as brain derived neurotrophic factor (BDNF; Dunbar et al., 2006; Rossignol et al., 2011), which may provide trophic support to remaining cells near the ischemic penumbra.

To determine the efficacy of BMMSCs in the chronic treatment phase, BMMSCs were transplanted into rats seven days after stroke was induced using the endothelin-1 (ET-1) injection method (Sharkey & Butcher, 1995). ET-1 is an endogenous hormone responsible for maintaining the homeostasis of vascular function. In a concentrated form, it can be injected directly into the brain, near a targeted blood vessel, causing the vessel to constrict and create a short-term blockage, which will naturally dilate and re-perfuse when the hormone dissipates. The ET-1 injection model of stroke results in fairly uniform infarcts, a low mortality rate, and low risk of post-surgical aphasia (Soleman et al., 2010), which makes this a suitable model for long-term studies.

In order to assess cognitive functioning without confounding motoric dysfunction, a modified version of the spatial operant reversal task (SORT), which was originally used to test pre-motoric cognitive deficits in a rodent model of Huntington’s disease (Fink et al., 2012), was used. Essentially, this task requires the animal to remember which of the two levers in the operant box activates the food delivery as the schedules of reinforcement are increased over the course of testing, while stability of motor functioning can be simultaneously assessed by measuring the overall number and rates of lever presses. Unlike the use of many instrumental tasks, such as the Morris water maze, in which it is more difficult to estimate the degree to which motoric dysfunction might interfere with assessment of cognition, particularly when testing rats that were given unilateral lesions, the SORT provides a more efficient and precise way to distinguish the relative contributions of the cognitive and motor components of the behavior being evaluated. Given this, we hypothesized the SORT would also be a useful tool in assessing cognitive recovery in rodent models of stroke, even several weeks after the insult. As such, the main goals of this study were to determine if: (1) rats injected with ET-1 would display cognitive and/or motoric deficits; (2) the SORT could accurately measure the cognitive deficits; and (3) injections of BMMSCs could attenuate these deficits.

2. Materials and methods

All procedures were approved by the Central Michigan University Animal Use and Care Committee, and followed the guidelines set forth by the National Institutes of Health.

2.1. Animals

Twenty-four male Sprague-Dawley rats (breeders from Charles River, offspring bred in-house), weighing between 250–325 grams, were used. Rats were dual housed when possible, in a room with a 12-hour reverse light cycle (lights on at 21:00). Rats were given access to food and water ad libitum, except during operant testing phases of the study, when they were food-restricted and maintained at 85% of their baseline weight.

2.2. Administration of endothelin-1

Rats were anaesthetized with a mixture of 5% isoflurane and O2, and maintained at 1–2% isoflurane throughout the surgery. The heads of the rats were shaved with a 3 cm wide Wahl® beard and moustache
The rat was euthanised via CO2 inhalation. Femur bones were removed and placed in the ear- and tooth-bars of the stereotaxic apparatus (David Kopf Instruments, Tejunga, CA). A 2.5 cm incision was made along the midline of the head using a sterile number 10 scalpel blade, to expose the temporal, parietal, and frontal lobes intersect of the left hemisphere, normally supplied by the middle cerebral artery (Sharkey & Butcher, 1995). This procedure produces an infarct in the area where the temporal, parietal, and frontal lobes intersect of the left hemisphere, normally supplied by the middle cerebral artery (Sharkey & Butcher, 1995). A mechanically-driven, 10 μL Hamilton syringe (Hamilton, Reno, NV) was lowered to −7.5 mm ventral from dura, and an injection of 4 μL of 100 pmol/μL ET-1 (Calbiochem, EMD Chemicals, San Diego, CA), was made at the rate of 0.5 μl per minute in 15 of the 24 rats (stroke group). The needle was left in place for three minutes, then raised 0.5 mm, and left for an additional three minutes, before being slowly withdrawn over the course of one minute to avoid aspirating the ET-1 back through the needle track. Nine of the 24 rats received identical surgeries, but received an equivalent volume of sterile saline (sham group). ET-1 surgeries, with the exception that the injections were divided into three groups: (1) ET-1-injected rats receiving injections of BMMSCs in Hanks Buffered Salt Solution (HBSS; Life Technologies-Invitrogen, Grand Island, NY) vehicle (Stroke + BMMSCs, n = 7), (2) ET-1-injected rats receiving injections of HBSS vehicle only (Stroke + Veh, n = 8); or (3) sham-injected rats receiving injections of HBSS vehicle only (Sham + Veh, n = 9). The transplant surgeries on these groups of rats followed the procedures used for the ET-1 surgeries, with the exception that the injections were made slightly posterior, medial, and dorsal to the coordinates used for the ET-1 delivery. Specifically, the burr-holes were drilled at the following coordinates: AP −0.4 mm and ML +3.0 mm from bregma, which was on the same horizontal plane as lambda. A10 μL Hamilton syringe was lowered to −5.5 mm ventral to the dura and injections of 200,000 BMMSCs in 1 μL of HBSS or the same volume of HBSS alone were made at a rate of 0.33 μl/min. The needle was left in place for 3 minutes after the completion of the injection, the needle was raised 0.1 mm, and a second 1 μL injection of 200,000 cells was made, for a total of 400,000 cells. The needle was again left in place for 3 minutes, then slowly withdrawn over the course of 1 minute. The application of bone wax, closure of the surgical site, and post-operative care were all performed as described for the previous surgery.

2.3. Stem cell preparation

Mesenchymal stem cells were prepared following the methods of Rossignol and colleagues (2011). Briefly, a 6 month-old female Sprague-Dawley donor rat was euthanised via CO2 inhalation. Femur bones were dissected from the body, and bone marrow was aspirated with a sterile syringe. BMMSCs were suspended in a solution of 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 10% horse serum (Invitrogen, Carlsbad, CA), and 10 mL Alpha Modified Eagle’s Medium (αMEM; Sigma, St Louis, MO) supplemented with 1% penicillin and streptomycin. BMMSCs were selected by plastic adherence (Azizi et al., 1998; Dominici et al., 2006; Rossignol et al., 2009; Rossignol et al., 2011), and cultured at 8000 cells/cm2, until they reached confluence, at which point they were passaged and replated. BMMSCs from this culture were transplanted into three cohorts of rats at passages 4–9. Flow cytometry was performed using a BD LSR-II Flow Cytometer (Part No. 642221, BD® Biosciences, San Jose, CA), and data analysed with FACSDiva Version 6.1.3 (BD® Biosciences, San Jose, CA) to confirm the mesenchymal stem cell surface marker CD90 was uniformly expressed in cohorts of cells corresponding to the ones that were transplanted in this study. Flow cytometry also confirmed that these cells also lacked expression of the hematopoietic marker CD45. The day of transplantation, BMMSCs were labelled, prior to passaging, with Hoechst 33342 (1/000; Thermo Scientific) in order to identify the location of cells post-transplantation.
2.5. Weight restriction

Seven days following the transplantation surgeries, the ad libitum feeding schedule was terminated and the food was removed from the cages, and replaced with daily rations of lab chow, such that, over the course of five days, rats reached 85% of their baseline weight. Once the goal weight was reached, only supplementary rations were given in the home cage to maintain this weight. During food restriction, rats were weighed and monitored daily, and supplementary food rations adjusted daily as needed. Food restriction continued until post-transplantation day 45, at which point rats were again given access to food ad libitum.

2.6. Operant training

All training and testing took place during the first two hours of the nocturnal phase, unless otherwise noted and followed the protocols set forth by Fink and colleagues (2012), using the same operant chambers and software. Rats were identified by ear notching and tail markings, and experimenters remained blind to group assignment throughout the course of testing. Only one training or testing session was performed each day for each rat, and all training and testing sessions lasted a maximum of 60 minutes, unless the daily maximum of 200 reinforcers was earned, which resulted in an early cessation of the trial.

Operant training began on post-transplant days 12 and 13, when the rats were auto-shaped to the food hopper by being placed in the operant chamber for 30 minutes, with only the house light on, and 30 sucrose reinforcer pellets (45 g each) randomly dropped into the food hopper. On post-transplant day 14, the rats were placed in the operant chamber for an eight-hour long acquisition phase with the cue light above the left lever illuminated, and were provided with water. During this task, only left lever presses provided reinforcers following a 30-second delay. The left lever remained as the goal (or active) lever during the remainder of the training period. This eight-hour acquisition training took place during the diurnal phase for all groups, between 00:00 and 08:00, and was the only trial in the study not performed during the nocturnal phase.

Beginning on post-transplant day 15, rats were placed in the operant boxes with the house light off, but the cue light above the left lever was illuminated, and was the only light illuminated during the remainder of the training phase. Rats were allowed to respond at a fixed ratio 1 (FR1) rate (every response on the left lever produces a reinforcer). This rate was gradually increased to an FR2, FR4, and FR8 as rats earned more reinforcers, with increases following each additional 10–15 reinforcers earned, until an FR5 (every 5th response reinforced) was achieved over the course of a 60-minute session. On day 16, the process was repeated, beginning at FR2 and gradually increasing until an FR10 was reached. On day 17, the process was repeated until an FR15 was reached. Finally on post-transplant days 18–19, rats were placed in the chamber with a set FR16 rate. All rats included in the study were able to respond at this criterion rate for two consecutive daily sessions by the end of the eight-day training day, and all rats learned this task at a similar rate. At the end of each daily session, rats were removed from the chamber, put into their home containers, and returned to their holding rooms, where they were separated to receive supplemental food. All equipment was wiped down with paper towels and Labsan 256Q disinfectant cleaner (Sanitation Strategies, Williamston, MI) between sessions. The purpose of this training was to prepare rats to respond at a progressive FR schedule, which comprised Phases 1 and 2 of the SORT.

2.7. Phase 1 of SORT

On post-transplant day 20, rats began the testing phase of the study, with one testing session daily. During Phase 1, the light above the left lever was illuminated, and only left lever presses were reinforced. This nine-day phase followed a progressive FR schedule, in the following pattern: FR2, FR4, FR8, FR16, FR32, FR64, FR128, FR256, FR512. Upon the completion of Phase 1, rats were given a day off, weighed, and fed supplemental food only.

2.8. Phase 2 of SORT

On post-transplant day 30, rats were returned to the operant boxes for testing Phase 2, which followed the same progressive FR schedule as in Phase 1, except the goal lever was reversed, so that the right goal light was illuminated and only right lever presses were reinforced.

2.9. Histology

Following behavioral testing, the rats were euthanized with an overdose sodium pentobarbital
(200 mg/kg; Med Pharmex, Pomona, CA) and transcardially perfused with 150 mL 0.1 M phosphate buffered saline (PBS) followed by 150 mL 4% paraformaldehyde (PFA) and brains were collected. The brains were post-fixed for 24 hours at 4°C in fresh 4% PFA, then moved to a 30% sucrose solution until saturated (approximately three days). When the brains sank to the bottom of the solution, they were removed and flash frozen in beaker of anhydrous methylbutane (Sigma, St. Louis, MO) on dry ice. The brains were sectioned at 30 μm, producing a series of six sections (with intervals at 180 μm) on a Vitekome Ultrapros S000 cryostat (GMI, Inc. Ramsey, MN), and the sections mounted directly onto positive-charged microscope slides (Globe Scientific, Paramus, NJ). Coronal sections at −0.22, −0.40, and −0.58 mm from bregma were stained with cresyl violet acetate. Slides were scanned using a Nikon Coolscan IV LS40 ED scanner (Nikon, Tokyo), and analyzed with SigmaScanPro image analysis version 5.0 (Jandel Scientific software, San Rafael, CA). Percentage of infarct volume was calculated following an established protocol (Swanson et al., 1990).

Immunolabeling was conducted using coronal sections at −0.25, −0.43, and −0.61 mm from bregma. Sections were first blocked against non-specific binding using 10% normal goat serum (NGS) in 0.1% Triton PBS solution and then labeled with rabbit anti-glial fibrillary acidic protein (GFAP; 1 : 1000; Millipore, Billerica, MA) for 18 hours at 4°C. Sections were then rinsed three times with 0.1 Molar PBS, and labeled with the fluorescent secondary antibody Alexa Fluor 488 (1 : 300 goat anti-rabbit, Invitrogen, Carlsbad, CA) in a solution with 0.1 Molar PBS and 0.1% Triton X-100 for 1 hour at room temperature (approximately 20°C). Slides were then rinsed with 0.1 Molar PBS and cover-slipped using VectaShield (Vector Labs, Burlingame, CA). Microscopic analysis was performed using a Zeiss Axiovert 200 M microscope, at 20x, with an epifluorescent setting (Carl Zeiss, Thornwood, NY). GFAP-labeled astrocytes were detected using a 488 nm filter cube, and Hoechst-labeled BMMSCs were detected using a 350 nm filter cube.

2.10. Statistics

The percentage of infarct volume of Stroke + Veh and Stroke + BMSSCs groups was analysed using a repeated measures ANOVA for changes across days and FR schedules following a previous protocol (Fink et al., 2012), followed by one-way ANOVAs and Protected Least Significant Difference post hoc tests when appropriate. The alpha levels were set at 0.05 for all analyses.

3. Results

3.1. Body weights

The one-way ANOVA revealed no significant between-groups differences in the average body weight during testing phases \( F(2,21)=0.525, p=0.599 \).

3.2. Characterization of BMSSCs

Analysis using flow cytometry of samples of the BMSSCs indicated that 85–99% of the cells expressed CD90 and 5–19% of the cells expressed CD45, adding further confirmation that these cells were predominately mesenchymal stem cells.

3.3. Operant task

A repeated measures ANOVA revealed no significant between-group differences on the number of incorrect lever presses \( F(2,21)=1.408, p=0.267 \), but a significant Group x Schedule interaction \( F(16,168)=2.108, p=0.010 \) during Phase 1 testing (Fig. 1A). One-way ANOVAs revealed that the groups differed at FR256 \( F(2,21)=3.953, p=0.035 \) and FR512 \( F(2,21)=3.887, p=0.037 \). Post-hoc analysis revealed that both Sham+Veh and Stroke+BMSSCs groups had fewer incorrect lever presses per second than the Stroke+Veh group at both FR256 and FR512. Importantly, there were no significant between-group differences in the total number of lever presses made during Phase 2 \( F(2,21)=4.35, p=0.026 \) during Phase 1, indicating the deficits observed were cognitive, rather than motoric in nature.

During Phase 2, the goal-lever reversal task, there was a significant between-group difference on the number of incorrect lever presses \( F(2,21)=5.157, p=0.015 \), with Stroke+Veh rats making more incorrect lever presses than either Stroke+BMSSCs or Sham+Veh rats (Fig. 1B). Again, there were no significant between-group differences in the total number of lever presses made during Phase 2 \( F(2,21)=1.91, p=0.157 \).
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Fig. 1. Rats given a stroke by injections of endothelin-1 and treated seven days later with transplants of bone-marrow-derived mesenchymal stem cells (Stroke+BMMSCs) had significantly fewer incorrect lever presses during Phase 1 (when pressing the left lever was the correct response; A) and during Phase 2 (when pressing the right lever was the correct response; B) than did rats that received vehicle injections following endothelin-1 administration (Stroke+Veh) in a spatial operant reversal task (SORT). The Stroke+BMMSCs rats performed at control levels in both phases of the SORT, when compared with rats given sham surgery followed by treatments with vehicle (Sham+Veh), despite the finding of no between-group differences in the total number of lever presses.

$p = 0.173$, indicating that these mnemonic deficits were not confounded by motoric dysfunction.

3.4. Percentage infarct volume

An independent samples Student’s t-test revealed that there were no significant differences between Stroke+BMMSCs and Stroke+Veh groups $t(13)=0.756$, $p = 0.463$ in the percentage of infarct volume.

3.5. Identification of injection sites and cell survival

Fluorescent microscopy revealed glial activation at the injection sites in Stroke+Veh and Stroke+BMMSCs rats (Fig. 2A and B) as well as near the ischemic penumbra in both groups (Fig. 2C and D). Hoechst-labeled BMMSCs were visible primarily at the injection site (Fig. 2B), with only a few BMMSCs detected near the ischemic penumbra in transplanted rats (Fig. 2D), indicating that a limited amount of cell migration to the penumbra may have occurred.

4. Discussion

The major findings of this study are that: (1) ET-1 induced long-term, stroke-related cognitive deficits in rats; (2) the cognitive deficits were detectable during high FR schedules of the SORT, without the confounding effects of motoric deficits; and (3) these deficits were attenuated by intracerebral injections of BMMSCs. Interestingly, the deficit in the SORT, as measured by the number of incorrect lever presses, emerged as the task became increasingly more difficult, and continued following the goal-lever reversal portion of the task. These differences remained relatively robust at post-transplant day 28–39 (days 35–46 post-stroke). The lack of differences between groups in the total number of lever presses indicates that these deficits were primarily cognitive, rather than motoric, in nature.

This is the first time, to our knowledge, that long-term cognitive-deficits have been reported at high FR schedules in a rodent model of stroke, and the first time an operant-task has been attempted in an ET-1 injected rat model of stroke. The fact that deficits were most severe at high FR schedules is interesting, particularly because previous studies using operant paradigms used a relatively low, FR20 schedule (Genovese et al., 1992) or a more complex delayed-matching and non-matching-to-position tasks (Higgins et al., 2002; Nelson et al., 1997) that required lower levels of physical activity.

When combined with previous work (Fink et al., 2012), our findings indicate that the SORT provides a sensitive measure of cognitive dysfunction that may
Fig. 2. Injection sites (arrows) had an increase in astrocyte labeling (green) in Stroke+Veh (A) and Stroke+BMMSCs (B) groups. Hoechst-labeled BMMSCs (blue) were visible near the injection site in transplanted rats (B). Evidence of activated astrocytes in the ischemic penumbra was also observed in both Stroke+Veh (C) and Stroke+BMMSCs (D) groups, with a limited number of Hoechst-labeled BMMSCs (small arrows) also visible near the penumbra in transplanted rats (D). Images at 20×; scale bar 100 μm for all images; infarct marked by asterisk (C-D). Brain atlas image from Paxinos & Watson (1986).
result of certain methodological differences between our study and that of Komatsu and colleagues (2010). For example, Komatsu and colleagues (2010) pretreated their stem cells prior to transplantation and also used a different stroke model (MCAO thread occlusion vs. ET-1), and stem cell delivery method (intravenous vs. surgical transplantation). The benefits noted in the present study, using unmodified BMSCs transplanted at 7 days, did result in improvements in cognitive function, but these benefits may have been due to the release of neurotrophic factors, such as BDNF (Dunbar et al., 2006; Rossignol et al., 2011), which has been shown to improve post-ischemic cognitive function in rats (Kiprianova et al., 1999), rather than cell replacement or any effect on the core area of the infarct. This assumption is supported by the fact that BMSCs showed evidence of survival, as measured by positive Hoechst labeling 49 days following transplantation.

In summary, the present findings demonstrate that the SORT can detect cognitive deficits several weeks following ET-1 induced stroke, well after spontaneous recovery is likely to occur. Importantly, these deficits can be attenuated by intracerebral injections of unaltered BMSCs, indicating that this approach holds significant potential for the treatment of stroke, even after several days following the ischemic event.

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