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ORIGINAL PAPER

Exosomes Secreted by the Cocultures of Normal and Oxygen–Glucose-Deprived Stem Cells Improve Post-stroke Outcome

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Abstract

Emerging stroke literature suggests that treatment of experimentally induced stroke with stem cells offered post-stroke neuroprotection via exosomes produced by these cells. Treatment with exosomes has great potential to overcome the limitations associated with cell-based therapies. However, in our preliminary studies, we noticed that the exosomes released from human umbilical cord blood-derived mesenchymal stem cells (MSCs) under standard culture conditions did not improve the post-stroke neurological outcome. Because of this apparent discrepancy, we hypothesized that exosome characteristics vary with the conditions of their production. Specifically, we suggest that the exosomes produced from the cocultures of regular and oxygen–glucose-deprived (OGD) MSCs in vitro would represent the exosomes produced from MSCs that are exposed to ischemic brain cells in vivo, and offer similar therapeutic benefits that the cell treatment would provide. We tested the efficacy of therapy with exosomes secreted from human umbilical cord blood (HUCB)-derived MSCs under in vitro hypoxic conditions on post-stroke brain damage and neurological outcome in a rat model of transient focal cerebral ischemia. We performed the TTC staining procedure as well as the neurological tests including the modified neurological severity scores (mNSS), the modified adhesive removal (sticky-tape), and the beam walking tests before ischemia and at regular intervals until 7 days reperfusion. Treatment with exosomes obtained from the cocultures of normal and OGD-induced MSCs reduced the infarct size and ipsilateral hemisphere swelling, preserved the neurological function, and facilitated the recovery of stroke-induced rats. Based on the results, we conclude that the treatment with exosomes secreted from MSCs at appropriate experimental conditions attenuates the post-stroke brain damage and improves the neurological outcome.

Keywords Stem cells · Exosomes · Ischemia · Reperfusion · Brain damage · Neurological recovery

Background

Stroke is one of the leading causes of death and a primary reason for disability worldwide (Katan and Luft 2018). Except the FDA-approved clot-busting drug, tissue-type plasminogen activator (tPA), currently there is no pharmacological treatment available to treat patients presenting with ischemic stroke within 4.5 h of symptom onset (Catanese et al. 2017). Considering the recent advances, mechanical thrombectomy is recommended in acute stroke patients with an Alberta Stroke Program Early Computed Tomography Score (ASPECTS) of > 5 (Gupta et al. 2012). Further, reduced edema, decreased prevalence of malignant infarctions, and improved clinical outcome was recently reported in patients with low initial ASPECTS after recanalization by mechanical thrombectomy (Broocks et al. 2019). Intra-arterial mechanical thrombectomy procedure could be a treatment choice in eligible patients present with acute ischemic stroke until 24 h of symptom onset (Powers et al. 2018). Speed to treatment is still invaluable because the delay in cerebral blood flow restoration beyond a critical time point cannot rescue the irreversibly damaged brain

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cells. The primary goal of thrombolytic therapy or mechanical thrombectomy is to reestablish the blood flow to the previously ischemic brain regions. Treatment to address the brain injury, which occurs due to ischemia and reperfusion before and after thrombolytic therapy or mechanical thrombectomy is still an unmet clinical need. Further, no clinically effective pharmacotherapies exist to date to facilitate the cellular functional recovery after an ischemic stroke. Cell therapy has emerged as a potential regenerative treatment to reduce post-stroke handicap (Kenmuir and Wechsler 2017). Several studies assessed the therapeutic potential of different types of stem cells as treatments for ischemic stroke, and the results of these studies are intriguing but conflicting (Marei et al. 2018).

Recent reports from our laboratory and others have demonstrated that the treatment with mesenchymal stem cells (MSCs), derived from the human umbilical cord blood (HUCB), reduced the post-stroke brain damage, inflammation, and apoptosis, improved the survival rate, and facilitated the neurological recovery of stroke-induced rats, rabbits, and canines (Chung et al. 2009; Lim et al. 2011; Kim et al. 2012; Zhu et al. 2014; Chelluboina et al. 2014). In addition, the administration of these cells in animal models of ischemic stroke prevented the post-ischemic induction of matrix metalloproteinases, downregulated the DNA damage-inducing genes, and upregulated the DNA repair genes (Chelluboina et al. 2016, 2017). The translational potential of MSCs treatment or any other cell-based therapies is limited because of the inability of cells to cross the blood–brain barrier to reach the ischemic brain region, the risk of occlusion in the microvasculature, and the potential risk of tumor formation in other organs (Jeong et al. 2011; Xiong et al. 2017).

The possible mechanisms of stem cell treatment-mediated protection in preclinical models include the differentiation of administered stem cells and the paracrine signaling. Multiple pieces of evidence suggest that both the differentiation and the paracrine-signaling mechanisms contribute to the positive stroke outcome in animal models of ischemic stroke (Chelluboina and Veeravalli 2015). In addition, it is well documented that the benefits of MSC treatment are primarily due to the paracrine effects via the release of exosomes, but not the cell replacement (Zhang and Chopp 2009; Moskowitz et al. 2010). Exosomes released from MSCs that contain a cargo of bioactive molecules including proteins, lipids, and genetic materials, play a vital role in intercellular communication (Valadi et al. 2007; Lotvall and Valadi 2007; Smalheiser 2007; Zomer et al. 2010; Katakowski et al. 2010; Record et al. 2011; Qi et al. 2016; Wahlgren et al. 2016). Exosomes released from MSCs are reported to improve the post-stroke outcome in rodent models of ischemic stroke (Xin et al. 2012; Xin et al. 2013a, b; Doeppner et al. 2015). We recently tested the efficacy of exosomes released from HUCB-MSCs under standard culture conditions in a rodent model of ischemic stroke and noticed that the treatment attenuated the post-stroke brain damage but did not improve the neurological recovery (Nalamolu et al. 2019). The efficacy of treatment with exosomes depends primarily on their content (Xin et al. 2013a, b; Zhang et al. 2017). The content of exosomes released from HUCB-MSCs under standard culture conditions could be different than their content when released under different experimental conditions. Therefore, we hypothesized that the exosomes produced from the cocultures of regular and OGD-induced MSCs in vitro would represent the exosomes produced from MSCs that are exposed to ischemic brain cells in vivo, and offer similar therapeutic benefits that the cell treatment would provide. In this study, we tested our hypothesis by determining the effect of treatment with exosomes secreted from HUCB-MSCs under in vitro hypoxic conditions on post-stroke brain damage and neurological function in a rat model of transient focal cerebral ischemia.

Methods

Stem Cells and Collection of Exosomes

Cryo-preserved human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) obtained from Vitro Biopharma (Golden, CO) were cultured in MSC-GRO low-serum complete MSC medium and maintained according to the manufacturer's protocol. MSCs cultured until their ninth passage were used for the collection of exosomes. For the collection of exosomes from hypoxic MSCs, cells were seeded at a density of 1.5 million cells per 100 mm plate containing MSC-GRO Low-Serum Complete MSC medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 12 h. For the collection of exosomes from MSCs under hypoxic conditions, cells were subjected to OGD for 3 h. After 3 h, culture media was replaced with glucose-containing DMEM media that has exosome depleted FBS. After 24 h, the culture medium was collected, and exosomes were extracted by using the Total Exosome Isolation kit (ThermoFisher scientific, USA) according to the manufacturer's protocol. For the collection of exosomes from the cocultures of normal and hypoxic MSCs, cells were seeded at a density of 0.5 million cells per 100 mm plate containing MSC-GRO Low-Serum Complete MSC medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 12 h. The cells were subjected to 3 h OGD followed by the addition of 1 million normal MSCs and replacement of culture media with glucose-containing DMEM media that has exosome depleted FBS. After 24 h, the culture medium was collected, exosomes were extracted using the Total Exosome Isolation kit. For the in vivo administration, estimation of surface

proteins present on exosomes was performed using Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Experimental Design, and the Induction of Focal Cerebral Ischemia and Reperfusion

Thirty-six healthy young adult male Sprague–Dawley rats were used in this study. Rats weighing 210 ± 10 g were procured (Envigo, USA) and housed in the Laboratory Animal Care Facility of the University of Illinois College Of Medicine at Peoria. After rats reached a weight of 240 ± 20 g, they were subjected to 2 h focal cerebral ischemia by right middle cerebral artery occlusion (MCAO) procedure followed by reperfusion as described earlier (Chelluboina et al. 2017). Rats were randomly assigned to EXO-hMSCs (ischemia-induced rats treated with exosomes obtained from MSCs that were subjected to hypoxic conditions), and EXO-MSCs + hMSCs (ischemia-induced rats treated with exosomes obtained from cocultures of normal and hypoxic MSCs) groups (Online Resource 1). The experimental design was shown as a schematic diagram in Fig. 1. Appropriate groups of rats were administered with exosomes (150 µg suspended in 0.5 mL sterile PBS/animal) that were collected under different experimental conditions via tail vein immediately after reperfusion. Mortality and body weight of all animals in the study were regularly recorded until 7 days reperfusion.

RT-PCR Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) from normal MSCs and 3 h OGD-induced MSCs. One µg of total RNA from each sample was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. RT-PCR was performed using the cDNAs obtained from various samples and the primers for human HIF-1α (primers: 5′-tgcatctccatctcctaccc-3′ and 5′-cgttagggcttcttggatga-3′) and the housekeeping gene GAPDH (primers: 5′-gagtcaacggatttggtcgt-3′ and 5′-gacaagcttcccgttctcag-3′) using GoTaq® Green Master Mix (Promega, USA) according to the manufacturer's protocol. RT-PCR was performed in C1000 Touch Thermocycler (Bio-Rad Laboratories, USA) using the following PCR cycle: [95 °C for 5 min, (95 °C for 30 s, 57 °C for 45 s, 72 °C for 45 s) × 40 cycles, and 72 °C for 5 min]. Final products were resolved on a 2% agarose gel containing ethidium bromide and visualized under UV light. Bands representing the mRNA expression of HIF-1α, and GAPDH were quantified using the Image J analysis (NIH) software.

Immunoblot Analysis

Exosomes obtained from MSCs under different experimental conditions were lysed, estimated for protein content, and subjected (20 µg protein/sample) to immunoblot analysis using CD63 antibody (Santa Cruz Biotechnology Inc. USA) as described earlier (Nalamolu et al. 2018b).

TTC Staining

2,3,5-Triphenyl tetrazolium chloride (TTC) staining procedure as well as the percent infarct size, and ipsilateral hemisphere swelling calculations were performed as described recently by our group (Nalamolu et al. 2019).

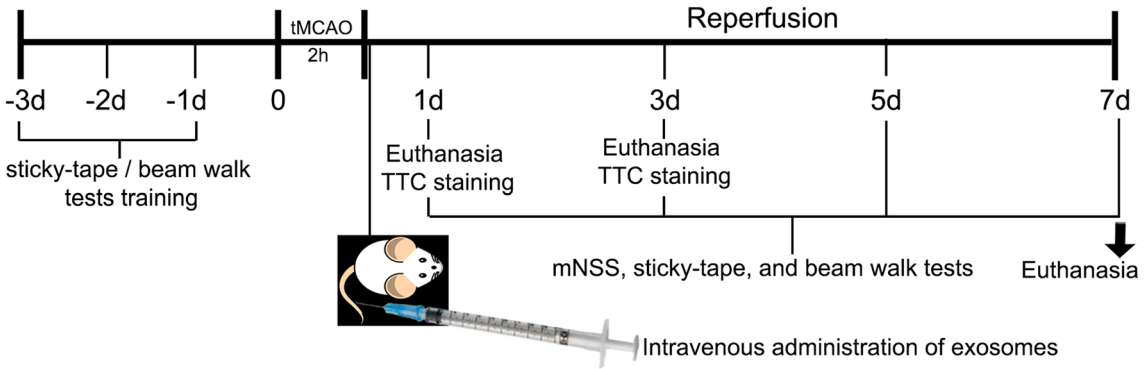

Fig. 1 Experimental design. Schematic diagram of the experimental design to evaluate post-stroke outcome. Rats were subjected to a right-transient MCAO for 2 h followed by 7 days reperfusion with neurological tests (mNSS, sticky-tape, and beam walking) on reperfusion days 1, 3, 5, and 7, and before ischemia. Appropriate cohorts were administered with exosomes collected under different experimental conditions intravenously via tail vein immediately after reperfusion. Rats were trained for sticky-tape and beam walk tests at least for 3 days prior to the induction of ischemia. Rats from few cohorts were euthanized at 1-day and 3-day reperfusion time points to perform TTC staining procedure

Neurological Evaluations

The modified neurological severity scores (mNSS) test, the modified adhesive removal (sticky-tape) test, and the beam walking test were performed as described recently by our group on rats of appropriate cohorts at regular intervals until 7 days reperfusion (Nalamolu et al. 2019).

Data Collection and Exclusion Criteria

Neurological evaluation tests were performed by the trained research personnel blinded to our treatments. We followed the recently reported exclusion criteria and accordingly excluded the animals or relevant data from analysis (Nalamolu et al. 2019).

Statistical Analysis

Statistical analysis of the data was performed using Graph Pad Prism software. Quantitative data of all the experiments were tested for normality and equality variances by F test or Bartlett's test. For RT-PCR analysis data, the difference between groups was analyzed by unpaired *t* test with Welch's correction. For infarct size and swelling, the differences between groups were analyzed by unpaired *t* test. We applied Welch's correction to the data that were not normally distributed or had unequal variances. Body weight, body weight gain, and neurological tests (mNSS, sticky-tape, and beam walking) data were analyzed for differences among the groups by one-way ANOVA followed by the Tukey's post hoc test. The data that were not normally distributed or had unequal variances were analyzed by the Kruskal–Wallis test followed by the Dunn's post hoc test. Results are expressed as mean ± SEM. Differences in the values were considered significant at $p < 0.05$.

Results

Stem Cells under Hypoxic Conditions Express HIF-1α

The representative bright-field images of normal and 3 h OGD-induced MSCs were shown in Fig. 2a. MSCs subjected to 3 h OGD appeared bulged and pumped up as compared to the flat and linear morphology noticed in control cells (Fig. 2a). The mRNA expression of HIF-1α, a well-known marker of hypoxia, was increased ($p < 0.001$) in OGD-induced MSCs as compared to the normal MSCs (Fig. 2b; Online Resource 2).

Typical Characteristics of Exosomes

We collected the exosomes from the MSCs that were subjected to OGD, and the cocultures of OGD-induced and normal cells. Immunoblot analysis demonstrated a high expression of CD63, a typical exosome marker, in the exosome samples collected at both the experimental conditions (Fig. 2c).

Exosome Treatment Prevents the Reduction in Post-Stroke Body Weight and Increases the Body Weight Gain

A total of three rats died during the study, and the data of five rats were excluded from the study. The mortality of 20–30% within a day after a 2 h MCAO procedure followed by reperfusion was reported in Sprague–Dawley rats (Hu et al. 2009,

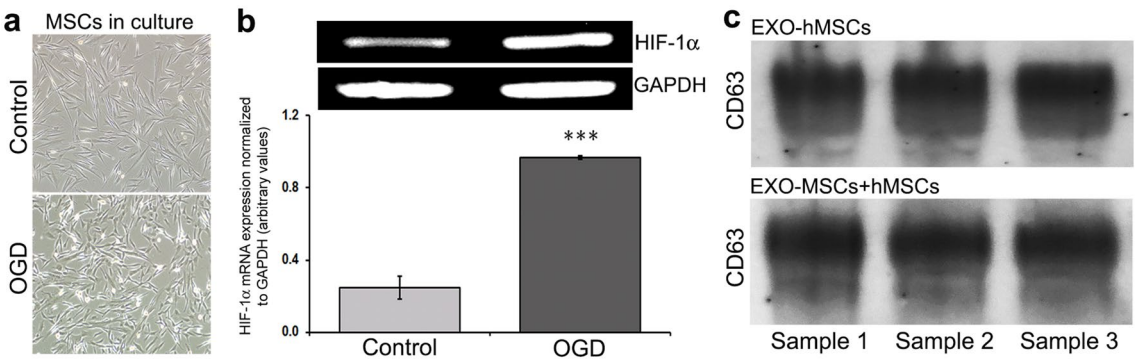

Fig. 2 Stem cells and OGD**. a** Representative bright-field images of the normal and 3-h OGD-induced HUCB-MSCs in culture. **b** RT-PCR analysis followed by agarose gel electrophoresis. Bands represent the mRNA expression of HIF-1α. GAPDH served as a loading control. Bar graph represent the densitometry analysis of HIF-1α bands (normalized to GAPDH). Histograms and error bars indicate the mean and the SEM, respectively. $n = 6$. ***$p < 0.001$ versus control. **c** Detection of exosomal marker CD63 expression by immunoblot analysis. *EXO-hMSCs* exosomes secreted by the hypoxic MSCs; *EXO-MSCs + hMSCs* exosomes secreted by the cocultures of normal and hypoxic MSCs

2011). As indicated by the 7-day post-stroke survival rate, the mortality of exosomes treated, stroke-induced rats were within the reported range (Fig. 3a). EXO-MSCs + hMSCs treatment but not the EXO-hMSCs treatment prevented the body weight reduction at 1-day reperfusion (Fig. 3b). Besides, EXO-MSCs + hMSCs treatment resulted in significant ($p < 0.01$ at 1 day reperfusion $p < 0.05$ at 3 day, 5 day, and 7 day reperfusion) increase of post-stroke body weight gain as compared to the vehicle-treated animals (data shown for comparison purposes) at all the reperfusion time points tested in this study (Fig. 3c). Results of statistical tests were presented in Online Resource 2. EXO-hMSCs treatment neither prevented the post-stroke body weight reduction at 1-day reperfusion nor increased the body weight gain at the reperfusion time points tested (Figs. 3b, c).

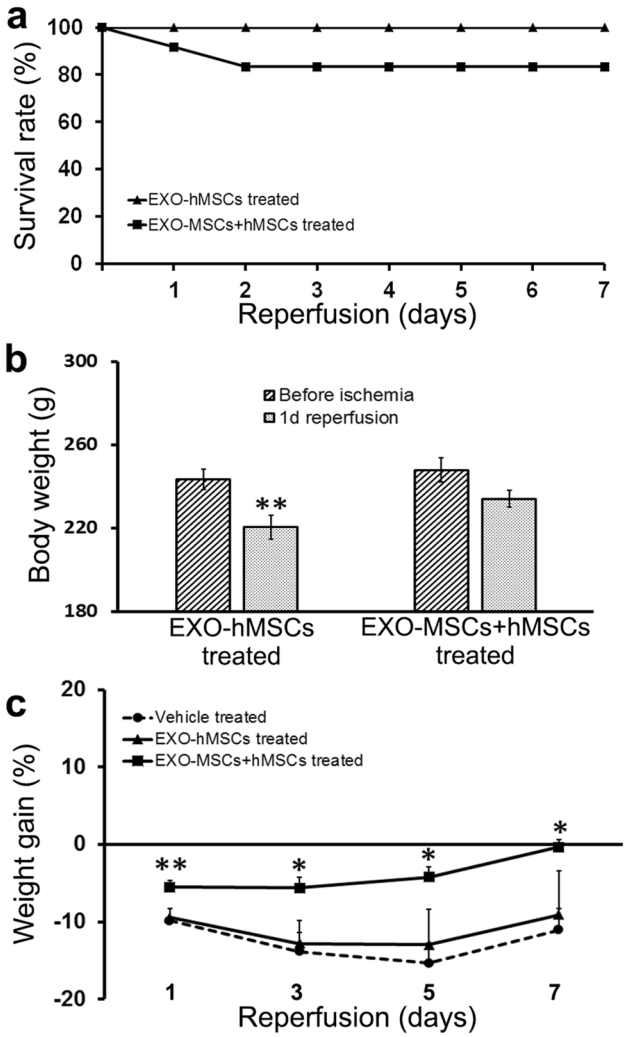

Fig. 3 Post-stroke mortality and body weight changes. **a** Line graph represents the percent survival rate of rats. $n = 10–12$. **b** Bar graph represents the reduction in body weight of rats at 1-day reperfusion. Histograms represent the mean, and error bars indicate SEM. $n = 8$. $^{**}p < 0.01$ versus before ischemia. **c** Line graph represents the percent body weight gain of rats. Error bars indicate SEM. $n = 8–9$. *EXO-hMSCs* exosomes secreted by the hypoxic MSCs; *EXO-MSCs + hMSCs* exosomes secreted by the cocultures of normal and hypoxic MSCs. Quantitative data of body weight gain obtained from various cohorts of rats in this study was compared with the appropriate data of vehicle-treated rats (Nalamolu et al. 2019). $^{*}p < 0.05$ versus vehicle-treated; $^{**}p < 0.01$ versus vehicle-treated

Exosomes Treatment Prevents the Post-stroke Brain Damage and Facilitates the Recovery

The mNSS scores of rats obtained from the mNSS test, which is a composite of motor, sensory, reflex and balance tests, provided a quantitative measure of the severity or degree of post-stroke injury. Before the induction of ischemia, rats from both the cohorts have a mean mNSS score of zero, which was expected in healthy animals. The mNSS scores of 6.38 ± 1.85 and 3.75 ± 1.27 at 1-day reperfusion indicated that the degree of post-stroke injury is moderate and mild in EXO-hMSCs and EXO-MSCs + hMSCs treated rats, respectively (Fig. 4). The mNSS scores of EXO-hMSCs and EXO-MSCs + hMSCs treated rats gradually decreased from 6.38 ± 1.85, and 3.75 ± 1.27 at 1-day reperfusion to 3.63 ± 1.09, and 0.86 ± 0.46 at 7 days reperfusion, respectively. The mNSS scores of exosome-treated rats were compared with the mNSS scores of vehicle-treated rats (data shown in Fig. 4 for comparison purposes). Although the mNSS score of EXO-hMSCs-treated rats were reduced as compared to the vehicle-treated rats at all the reperfusion time points, the reduction was not significant. Interestingly,

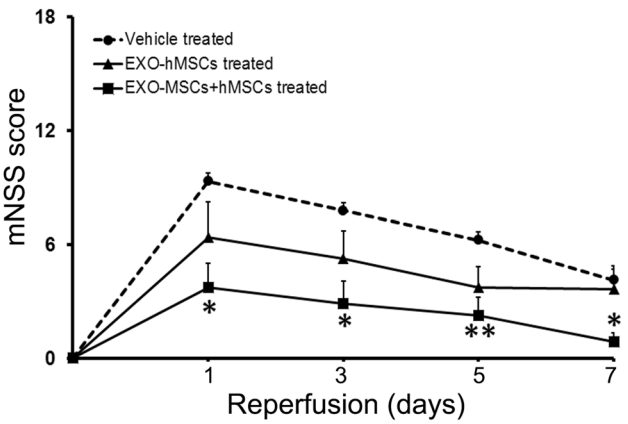

Fig. 4 Assessment of the severity of post-stroke injury and recovery. Severity of the post-stroke injury at 1-day reperfusion including the degree of recovery during reperfusion was assessed at regular intervals by the mNSS test in rats subjected to 2-h ischemia followed by 7 days of reperfusion. Quantitative data of mNSS scores obtained from various cohorts of rats in this study was compared with the appropriate data of vehicle-treated rats (Nalamolu et al. 2019). Error bars indicate SEM. $n = 8–9$. *EXO-hMSCs* exosomes secreted by the hypoxic MSCs; *EXO-MSCs + hMSCs* exosomes secreted by the cocultures of normal and hypoxic MSCs. $^{*}p < 0.05$ versus vehicle-treated; $^{**}p < 0.01$ versus vehicle-treated

the mNSS score of EXO-MSCs + hMSCs treated rats was significantly ($p < 0.05$) reduced as compared to the vehicle-treated rats at 1-day reperfusion (Fig. 4; Online Resource 2). These results indicated that the treatment with EXO-MSCs + hMSCs immediately after reperfusion mitigated the post-stroke brain damage. Further, the mNSS scores of EXO-MSCs + hMSCs treated rats were reduced significantly ($p < 0.05$ at 3 day and 7 day reperfusion; $p < 0.01$ at 5 day reperfusion) as compared to the respective scores of vehicle-treated rats at all the reperfusion time points tested (Fig. 4; Online Resource 2). These results indicated that EXO-MSCs + hMSCs treatment facilitated post-stroke neurological recovery.

Further, we tested the effect of EXO-MSCs + hMSCs treatment on post-stroke brain damage by performing the TTC staining at 1-day and 3-day reperfusion. Representative TTC stained images of EXO-MSCs + hMSCs treated rats euthanized at 1-day and 3-day reperfusion were shown in Fig. 5a. The degree of percent infarct size and swelling in EXO-MSCs + hMSCs treated rats was compared with the percent infarct size and swelling of untreated, ischemia-induced rats that we reported earlier (Chelluboina et al. 2015; Nalamolu et al. 2018b). EXO-MSCs + hMSCs treatment reduced the percent infarct size to 27.02 ± 7.72, and 15.85 ± 6.88 at 1-day, and 3-day reperfusion as compared to 58.9 ± 4.27, and 54.76 ± 6.22, respectively, in untreated, ischemia-induced rats (Fig. 5b). The decrease in infarct size in EXO-MSCs + hMSCs treated rats at both the reperfusion time points was significant ($p < 0.01$ at 1 day reperfusion; $p < 0.001$ at 3 day reperfusion) as compared to the untreated, ischemia-induced rats (Fig. 5b; Online Resource 2). Similarly, EXO-MSCs + hMSCs treatment reduced the percent swelling to 12.64 ± 1.2, and 4.07 ± 0.89 at 1-day and 3-day reperfusion as compared to 16.32 ± 2.03, and 34.86 ± 3.37, respectively, in untreated, ischemia-induced rats (Fig. 5b). The decrease in swelling in EXO-MSCs + hMSCs treated rats as compared to the untreated, ischemia-induced rats was not significant at 1-day reperfusion, but significant ($p < 0.001$) at 3-day reperfusion (Fig. 5b; Online Resource 2).

Exosome Treatment Reduces the Post-stroke Somatosensory Dysfunction

The sticky-tape ratio of rats obtained from the sticky-tape test provided a quantitative measure of the post-stroke somatosensory function. Before the induction of ischemia, rats from both the cohorts have a mean sticky-tape ratio of one, which was expected in healthy animals. The sticky-tape ratio of 0.37 ± 0.16 and 0.72 ± 0.15 at 1-day reperfusion in EXO-hMSCs and EXO-MSCs + hMSCs treated rats, respectively indicated that the post-stroke somatosensory function was compromised (Fig. 6a). The sticky-tape ratios of EXO-hMSCs, and EXO-MSCs + hMSCs treated rats gradually

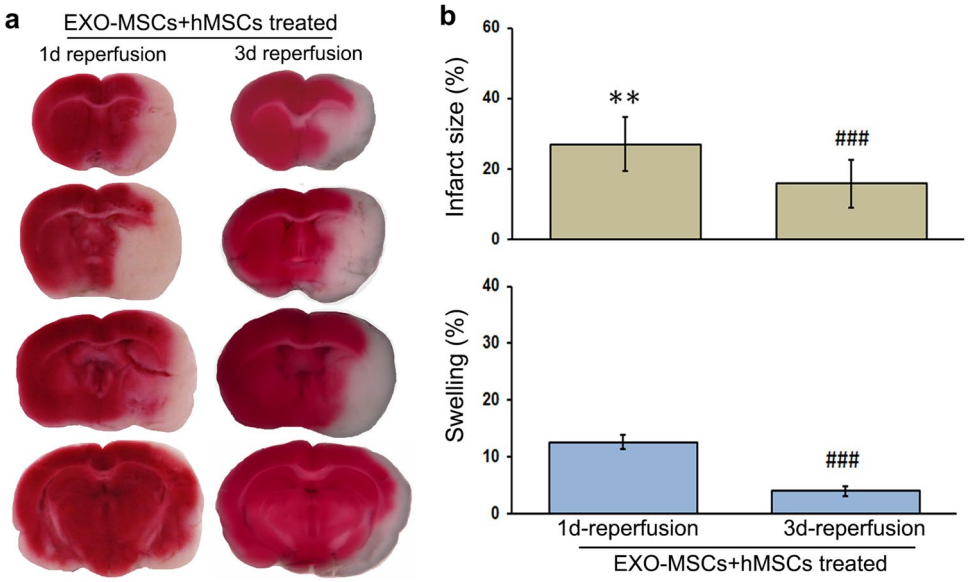

Fig. 5 Effect of treatment with exosomes on infarct size and swelling. **a** Representative TTC stained images of rat coronal brain sections at 1-day and 3-day reperfusion subsequent to a 2-h focal cerebral ischemia in rats. **b** Bar graphs represent the quantitative data of infarct size and ipsilateral hemisphere swelling. Histograms and error bars indicate the mean and the SEM, respectively. $n = 6$. *EXO-MSCs + hMSCs* exosomes secreted by the cocultures of normal and hypoxic MSCs. Quantitative data of infarct size and swelling obtained from EXO-MSCs + hMSCs treated rats in this study was compared with the appropriate data of untreated, ischemia-induced rats that we reported earlier (Chelluboina et al. 2015; Nalamolu et al. 2018b). $^{**}p < 0.01$ versus untreated ischemia-induced rats euthanized at 1-day reperfusion; $^{###}p < 0.001$ versus untreated ischemia-induced rats euthanized at 3-day reperfusion

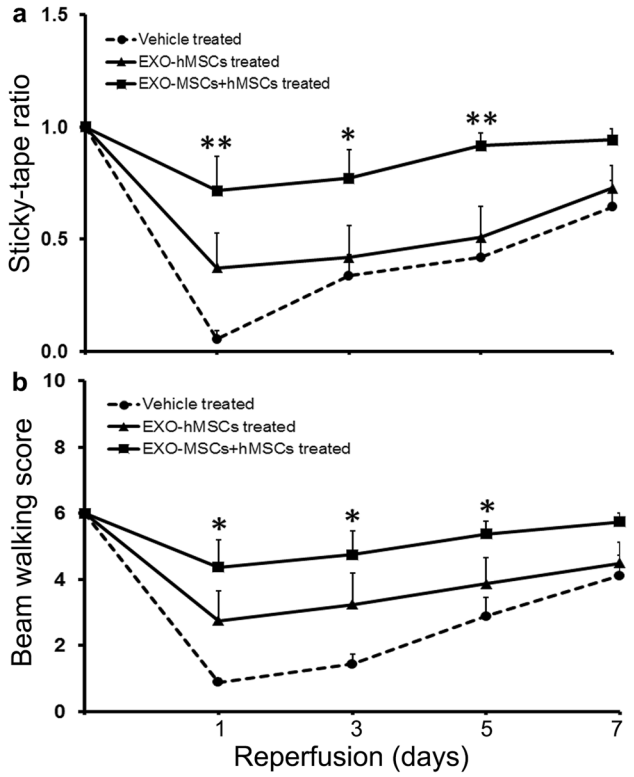

Fig. 6 Assessment of the post-stroke somatosensory dysfunction and deficits in coordination and integration of motor movement. Somatosensory dysfunction (**a**), and deficits in coordination and integration of motor movement (**b**) at 1-day reperfusion including the degree of recovery during reperfusion were assessed at regular intervals by the sticky-tape test, and the beam walking test, respectively, in rats subjected to 2-h ischemia followed by 7 days reperfusion. Quantitative data of sticky-tape ratio and beam walking scores obtained from various cohorts of rats in this study was compared with the appropriate data of vehicle-treated rats (Nalamolu et al. 2019). Error bars indicate SEM. $n = 7–9$. *EXO-hMSCs* exosomes secreted by the hypoxic MSCs, *EXO-MSCs + hMSCs* exosomes secreted by the cocultures of normal and hypoxic MSCs. *$p < 0.05$ versus vehicle-treated; **$p < 0.01$ versus vehicle-treated

increased from 0.37 ± 0.16, and 0.72 ± 0.15 at 1-day reperfusion to 0.73 ± 0.1, and 0.94 ± 0.05 at 7 days of reperfusion, respectively. The sticky-tape ratios of exosomes treated rats were compared with the sticky-tape ratio of vehicle-treated rats (data shown in Fig. 6a for comparison purposes). Although the sticky-tape ratio of EXO-hMSCs treated rats was increased as compared to the vehicle-treated rats at 1-day reperfusion, the increase was not significant. Interestingly, the sticky-tape ratio of EXO-MSCs + hMSCs treated rats was significantly ($p < 0.01$) increased as compared to the vehicle-treated rats at 1-day reperfusion (Fig. 6a; Online Resource 2). These results indicated that the treatment with EXO-MSCs + hMSCs reduced the degree of post-stroke somatosensory dysfunction. Although the sticky-tape ratios of EXO-hMSCs treated rats were increased as compared to the vehicle-treated rats at the remaining reperfusion time points, the increase was not significant. As expected, the sticky-tape ratios of EXO-MSCs + hMSCs treated rats were increased significantly ($p < 0.05$ at 3 day reperfusion; $p < 0.01$ at 5 day reperfusion) as compared to the respective ratios of vehicle-treated rats (Fig. 6a; Online Resource 2). These results indicated that EXO-MSCs + hMSCs treatment improved the post-stroke somatosensory function.

Exosome Treatment Preserves the Motor Function After Ischemic Stroke

The beam walking scores of rats obtained from the beam walking test provided a quantitative measure of the coordination and integration of motor movement. Before the induction of ischemia, rats from both the cohorts have a beam walking score of six, which was expected in healthy animals. The beam walking score of 2.75 ± 0.9 and 4.38 ± 0.83 at 1-day reperfusion in EXO-hMSCs, and EXO-MSCs + hMSCs treated rats, respectively indicated that the post-stroke motor function was compromised (Fig. 6b). The beam walking scores of EXO-hMSCs, and EXO-MSCs + hMSCs treated rats gradually increased from 2.75 ± 0.9, and 4.38 ± 0.83 at 1-day reperfusion to 4.5 ± 0.64, and 5.75 ± 0.27 at 7 days of reperfusion, respectively. The beam walking scores of exosome treated rats were compared with the beam walking score of vehicle-treated rats (data shown in Fig. 6b for comparison purposes). Although the beam walking score of EXO-hMSCs treated rats was increased as compared to the vehicle-treated rats at 1-day reperfusion, the increase was not significant. Interestingly, the beam walking score of EXO-MSCs + hMSCs treated rats was significantly ($p < 0.05$) increased as compared to the vehicle-treated rats at 1-day reperfusion (Fig. 6b; Online Resource 2). These results indicated that the treatment with EXO-MSCs + hMSCs immediately after reperfusion reduced the degree of post-stroke deficits in coordination and integration of motor movement. Although the beam walking scores of EXO-hMSCs treated rats were increased as compared to the vehicle-treated rats at the remaining reperfusion time points, the increase was not significant. As expected, the beam walking scores of EXO-MSCs + hMSCs treated rats were increased significantly ($p < 0.05$ at 3 day and 5 day reperfusion) as compared to the respective scores of vehicle-treated rats (Fig. 6b; Online Resource 2). These results indicated that EXO-MSCs + hMSCs treatment improved the post-stroke motor function.

Discussion

In this study, we demonstrate for the first time that the exosomes released from the cocultures of normal and hypoxic HUCB-MSCs mitigate post-stroke brain damage,

preserve neurological functions, and significantly improve recovery. Cell therapy may provide a promising new treatment for ischemic stroke. Recent studies in stroke patients have revealed the benefits of cell-based therapies (Kenmuir and Wechsler 2017). Although several preclinical studies suggest that cell-based therapies are effective in improving post-stroke functional outcome, further investigations are required to optimize the type of cells used, delivery methods, dosing, and timing of delivery. We and others have shown the benefits of treatment with HUCB-MSCs on post-stroke outcome in rodent and non-rodent models of ischemic stroke (Chung et al. 2009; Lim et al. 2011; Kim et al. 2012; Zhu et al. 2014; Chelluboina et al. 2014, 2016, 2017). The primary mechanism of protection underlying the MSCs treatment could be mediated through the exosomes released from the cells (Zhang and Chopp 2009; Moskowitz et al. 2010). Exosomes are the endosome-derived small-membrane vesicles that are released into extracellular fluids by cells. The complex cargo of proteins and genetic materials in exosomes participates in multiple biochemical and cellular processes, an important attribute required for treatment of post-stroke pathogenesis that involves various injury mechanisms, pathways, and molecules. The efficacy of treatment with exosomes depends mainly on the content, dose, and availability of exosomes at the target site. For example, in this study, exosomes were administered intravenously at a dose of 150 µg/animal. Administration of these exosomes intra-arterially into the right internal carotid artery via the common carotid artery after removal of the monofilament suture could have increased their availability in the ischemic brain and offered a higher degree of post-stroke protection and neurological recovery. If the benefits of treatment with MSCs are mediated through exosomes, direct treatment with exosomes could be the best approach to overcome the limitations associated with cell-based therapies.

The interest in the use of exosomes as potential therapeutic tools to treat stroke and possibly other diseases is increasing. The administration of exosomes secreted from MSCs of two independent human bone marrow lines improved the post-stroke motor coordination (Doeppner et al. 2015). In the contrary, the administration of exosomes secreted from MSCs of rat bone marrow did not show any significant improvement in the mNSS score at 7 days of reperfusion (Xin et al. 2013a). Similarly, the administration of exosomes secreted from HUCB-MSCs under standard culture conditions failed to improve the post-stroke somatosensory and motor functions, although reduced the infarct size (Nalamolu et al. 2019). The discrepancy in results of these studies could be attributed to the source of MSCs, the dose of exosomes, and primarily the experimental conditions at which the exosomes are collected from cells.

The MSCs could behave differently under different experimental conditions. For example, treatment with HUCB-MSCs induced apoptosis in cancer cells but inhibited apoptosis when given to spinal cord-injured or stroke-induced rats (Dasari et al. 2009; Gondi et al. 2010; Chelluboina et al. 2014). Based on our previous studies from the past decade involving HUCB-MSCs, we believe that the HUCB-MSCs are capable of producing even opposite effects, and the impact of HUCB-MSCs treatment depends primarily on the microenvironment they were in. The composition of exosomes released from cells depends on the cellular condition (Eldh et al. 2010). The molecular constituents of exosomes varies with cell origin, and physiological or pathological conditions the cells were exposed to. In addition, the content of exosomes released by cells could be different under different experimental conditions. For example, the microRNA-133b levels of exosomes secreted from rat femur- and tibia marrow-derived MSCs exposed to normal rat brain tissue extracts versus rat post-ischemic brain tissue extracts were significantly different (Xin et al. 2012).

As expected, in this study, treatment with exosomes secreted from the cocultures of normal and hypoxic MSCs offered significantly greater protection than the treatment with exosomes secreted from the hypoxic MSCs. As compared to the corresponding vehicle treatment, EXO-MSCs + hMSCs treatment, but not the treatment with EXO-hMSCs, prevented the post-stroke reduction of body weight, sticky-tape ratio, beam walking score, and the post-stroke elevation of mNSS score as well as facilitated the body weight gain, somatosensory, and motor functions until 7 days reperfusion in stroke-induced young adult male rats. In addition, EXO-MSCs + hMSCs treatment attenuated the infarct size and swelling of the ipsilateral hemisphere. The therapeutic effect of EXO-MSCs + hMSCs treatment in this study is in agreement with the therapeutic effect offered by the direct HUCB-MSCs treatment (Chung et al. 2009; Kim et al. 2012; Nalamolu et al. 2018a). It could be the difference in type and expression levels of microRNAs in the exosomes collected under co-culture conditions that would have contributed to the therapeutic benefit. The differences in the protein content of exosomes collected at different experimental conditions could also have contributed to the differences in their efficacy. In addition, the different structural composition of exosomes collected from stem cells, which were exposed to various experimental conditions may impact their pharmacokinetic properties. Caveats of this study include testing the efficacy of exosomes at a single dose based on the amount of proteins expressed on the surface of exosomes as well as the lack of experiments to determine the molecular constituents of exosomes and their presence in the ischemic brain. Unlike cells, the small size of exosomes and the expression of adhesive molecules on their membrane, however, facilitates their entry into the ischemic brain (Clayton et al. 2004). Our future studies will determine the molecular constituents of exosomes released

by HUCB-MSCs under normal and hypoxic conditions. This information could provide significant clues for the development of potential stroke therapies in the future.

Conclusions

Based on the results of this study, we conclude that the treatment with exosomes released from the cocultures of normal and hypoxic HUCB-MSCs attenuate the post-stroke brain damage and facilitate the neurological recovery of stroke-induced young adult male rats. Efficacy of treatment with exosomes primarily depends on the content of exosomes, which in turn depends on the cell's microenvironment while secreting the exosomes. We believe that the exosomes that are being released from the cocultures of normal HUCB-MSCs and hypoxic brain cells could offer more therapeutic benefit than the exosomes tested in this study.

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Author Contributions KKV conceived and designed the study. KKV, KRN, IV and AM performed the experiments and collected the data. KKV and KRN analyzed the data. KKV wrote the paper. JDK, DMP, DZW, and AK reviewed and edited the manuscript. All authors read and approved the manuscript. KRN and IV contributed equally to this work.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no competing interests.

Ethical Approval The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois College of Medicine at Peoria approved all surgical interventions and post-operative animal care. All the animal experiments conducted were in accordance with the approved animal protocol and the IACUC guidelines.

Informed Consent Not applicable to this study.

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