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Exosomes Treatment Mitigates Ischemic Brain Damage but Does Not Improve Post-Stroke Neurological Outcome

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Abstract

Background/Aims: Recent studies demonstrated that the treatment with mesenchymal stem cells (MSCs) obtained from the human umbilical cord blood improved survival, reduced brain damage, prevented apoptosis, suppressed inflammatory responses, downregulated the DNA damage-inducing genes, upregulated the DNA repair genes, and facilitated neurological recovery in stroke-induced animals. Emerging stroke literature supports the concept that the exosomes released from MSCs are the primary biological principles underlying the post-stroke neuroprotection offered by MSCs treatment.

Methods: Because the treatment with exosomes has a great potential to overcome the limitations associated with cell-based therapies, we tested the efficacy of exosomes secreted from HUCB-MSCs under standard culture conditions on post-stroke brain damage and neurological outcome in a rat model of ischemic stroke by performing TTC staining as well as the modified neurological severity scores, modified adhesive removal, beam-walking, and accelerating Rotarod performance tests before ischemia and at regular intervals until seven days reperfusion.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

Results: Exosomes treatment attenuated the infarct size. Treatment with exosomes did not affect the post-stroke survival rate and body weight changes, but exacerbated the somatosensory and motor dysfunction and adversely affected the natural recovery that occurs without any treatment.

Conclusion: Treatment with exosomes secreted from HUCB-MSCs under standard culture conditions attenuates the ischemic brain damage but does not improve the post-stroke neurological outcome.

Keywords

Ischemia; Reperfusion; Stroke; Recovery; Stem cells; Exosomes

Introduction

The emerging literature on stroke research demonstrates the therapeutic potential of mesenchymal stem cell (MSC)-derived exosomes in preclinical rodent models of ischemic stroke. Studies reported that exosomes are secreted by all cells of rodent and human brains [1–3]. The size of exosomes varies from 30 nm to 100 nm in diameter [4–8]. Exosomes contain a cargo of small molecules such as proteins, lipids, and genetic materials, and are generally enriched with Alix, CD9, CD63, CD81, HSP70, annexins, tubulin, actin, actin-binding proteins, etc. [5, 7, 9–12]. Exosomes may be internalized by other cells by membrane fusion, endocytosis, or cell-type specific phagocytosis [13–15]. Exosomes are known to play a vital role in intercellular communication. Once released from a cell, exosomes facilitate cell-to-cell communication and alter the function of recipient cells by transferring the proteins and genetic materials such as mRNAs, microRNAs, rRNAs, long non-coding RNAs (lncRNAs), DNAs to other cells [10, 16–23].

Recent studies from our laboratory and others have demonstrated that the administration of MSCs derived from human umbilical cord blood (HUCB) improved survival, reduced brain damage, prevented apoptosis, suppressed inflammatory responses, and facilitated neurological recovery in stroke-induced rats [24]. We also reported that the administration of HUCB-MSCs to stroke-induced rats prevented the post-ischemic induction of matrix metalloproteinases, downregulated the DNA damage-inducing genes, and upregulated the DNA repair genes without disturbing the endogenous defense mechanisms [25, 26]. The translational potential of HUCB-MSCs or any other cell-based therapies is associated with several limitations including the inability of cells to cross the blood-brain barrier and reach ischemic region, the risk of occlusion in the microvasculature, and potential risk of tumor formation in other organs. In our previous studies, for the administered cells to cross the blood-brain barrier and reach the ischemic brain, we administered the cells to stroke-induced rats at one-day after reperfusion after two-hour ischemia. Although this approach improved the post-stroke outcome, it did not address the damage that occurred within a day after transient focal cerebral ischemia and reperfusion because the treatment was instituted one-day after reperfusion. The therapeutic effects produced by HUCB-MSCs in these animal models could be attributed to either the replacement of injured tissue with the differentiated HUCB-MSCs or the bioactive molecules secreted by HUCB-MSCs. Although the initial concept of therapy with stem cells was aimed at replacing dead tissue, recent studies suggest that the treatment with stem cells improves the post-stroke outcome by stem cell-secreted

factors. It is well documented that the MSCs treatment-mediated neurorestorative effects after stroke are primarily due to the paracrine effects of the administered MSCs on brain parenchymal cells via the release of exosomes and their cargo, but not the cell replacement [27, 28]. Emerging data indicate that the exosomes released from the multipotent MSCs derived from femur and tibia marrow of rats and bone marrow of humans provide therapeutic benefits in both rat and mouse models of ischemic stroke [29–32].

In our previous study, the possibility of HUCB-MSCs treatment offered therapeutic benefit due to the replacement of injured brain tissue would be highly unlikely because only a small portion of injected cells may reach the damaged site, survive, and differentiate into various types of brain cells. Therefore, we hypothesize that the exosomes derived from HUCB-MSCs could attenuate brain damage and improve the neurological outcome in stroke-induced animals. As per the Stroke Treatment Academic Industry Roundtable criteria, we performed these initial studies in healthy young adult male rodents.

Materials and Methods

Stem cells and collection of exosomes

Cryo-preserved HUCB-MSCs obtained from Vitro Biopharma (Golden, CO) were cultured in MSC-GRO low serum complete MSC medium according to the manufacturer's protocol. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 with a change of media twice a week. When the cells reached about 90% confluence, they were detached and sub-cultured using Trypsin-EDTA. HUCB-MSCs were used only to the 9^{th} passage and not beyond. Approximately 1.5 million cells were seeded in a 100 mm plate containing MSC-GRO Low Serum Complete MSC medium and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After 12 h, the culture medium was replaced with 6 mL of DMEM media containing exosome depleted FBS. After 24 h, the culture medium was collected, and exosomes were extracted by using the Total Exosome Isolation kit (Thermo-Fisher scientific, USA) according to the manufacturer's protocol. Briefly, the culture medium was centrifuged at 2000 x g for 30 min, and the supernatant was collected. 500 μL of total exosome isolation reagent per 1 mL of the supernatant was added and incubated overnight at 4 °C. After the overnight incubation, the culture medium was centrifuged for 1 h at 10, 000 x g. The supernatant was aspirated, and the pellet was resuspended in a suitable volume of sterile PBS.

Animals and experimental design

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. The housing conditions included a 12-hour light/dark cycle, controlled temperature and humidity, and free access to food and water. Rats were randomly assigned to two cohorts and subjected to the transient focal cerebral ischemia and reperfusion followed by treatment. 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. The experimental design was shown as a schematic diagram in Fig. 1. Briefly, rats were subjected to 2h ischemia

followed by seven days reperfusion with neurological evaluations before ischemia and on reperfusion days 1, 3, 5, and 7. Cohorts were administered with either vehicle or exosomes immediately after reperfusion. A subset of animals treated with exosomes was euthanized at one-day reperfusion for TTC staining. Mortality and body weight of all animals in the study were recorded at regular intervals until seven days reperfusion. All the animal experiments conducted were in accordance with the IACUC guidelines and the approved animal protocol.

Induction of transient focal cerebral ischemia and reperfusion

After the animals reached a weight of 240±20 g, they were anesthetized with isoflurane (3–4%) and subjected to right MCAO procedure by inserting a silicone rubber coated monofilament suture (Doccol Corporation, California) into the internal carotid artery via the external and common carotid arteries as described earlier [26]. Reperfusion was initiated two hours after MCAO by removing the monofilament suture from the internal carotid artery. A knot with a silk suture was tied on the external carotid artery at bifurcation to stop bleeding. The skin was sutured to close the neck incision. Rats from both the cohorts subjected to MCAO procedure were administered the recommended doses of buprenorphine, and cefazolin to mitigate pain and prevent infection. We did not administer any immunosuppressants to rats in this study.

Exosomes treatment

The protein content of the exosomes in the pellet was measured by using the Pierce ® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Rats that were randomly allocated to two cohorts (n = 15 per group) were administered exosomes (150 μg/animal) in 0.5 mL sterile phosphate-buffered saline (PBS) or PBS alone (vehicle) intravenously via tail vein immediately after reperfusion.

Immunoblot analysis

Exosomes obtained from HUCB-MSCs were subjected to immunoblot analysis using antibodies for CD9 (monoclonal; catalog # SC13118), and CD63 (monoclonal; catalog # SC365604) followed by HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using chemiluminescence ECL Western blotting detection reagents (Bio-Rad Laboratories, USA).

TTC staining

Rats treated with exosomes were deeply anesthetized with pentobarbital and decapitated. Brains were removed, placed in adult rat brain matrix (Kent Scientific Corporation, USA), frozen at approximately −70 °C for about 8–10 min, sliced into 2 mm thick coronal sections. Coronal brain sections were incubated in 2% 2, 3,5-triphenyl tetrazolium chloride (TTC) solution for 30–45 min in the dark followed by the capture of images using the Olympus SZX12 research stereomicroscope. The areas of ischemic and non-ischemic regions of the ipsilateral hemisphere as well as the contralateral hemisphere of each section were traced and measured using Image J analysis software (NIH). Total volumes of each region of rat brains were calculated. The percent infarct size in each rat was calculated by using the formula, infarct size (%) = {(volume of the contralateral hemisphere)-(volume of the non-

ischemic ipsilateral hemisphere)} x 100 / volume of the contralateral hemisphere. This formula accounts for the possible interference of brain edema on infarct volume. Ipsilateral hemisphere swelling in each rat was calculated by using the formula, swelling (%) = {(volume of the ipsilateral hemisphere)-(volume of the contralateral hemisphere)} x 100 / volume of the contralateral hemisphere. Effect of treatment with exosomes on infarct size and ipsilateral hemisphere swelling was determined by comparing the results of this study with the data of untreated, ischemia-induced rats that we reported earlier [33].

Modified neurological severity scores (mNSS) test

The mNSS test is the standard and globally accepted method to assess the severity of post-stroke injury and recovery [34]. This test is a composite of motor, sensory, reflex and balance tests. A cumulative score from all the tests determines the severity of the injury. An mNSS score of 13–18 indicates severe injury, 7–12 indicates moderate injury and 1–6 means mild injury. The mNSS test was performed on rats of both the cohorts before ischemia and at regular intervals (1d, 3d, 5d, and 7d) until seven days reperfusion.

Modified adhesive removal (sticky-tape)test

Sticky-tape test is an assessment of post-stroke somatosensory dysfunction [35]. This test can reliably quantify the degree of focal sensory impairment without prolonged pre-training. Sticky-tape test was performed on rats of both the cohorts before ischemia and at regular intervals (1d, 3d, 5d, and 7d) until seven days reperfusion. In this test, a sticky-tape was wrapped around the ipsilateral and contralateral forepaw of a rat and attempts made by the animal to remove the tape were recorded. Both the affected forelimb and the contralateral forelimb were tested with a minimum of three trials for each limb at each time point. An average of three trails from both ipsilateral and contralateral forepaw was calculated. Sticky-tape ratio was calculated by using the formula, Sticky-tape ratio = Average of three trials of the affected forelimb / Average of three trials of the contralateral forelimb. Before the induction of ischemia, rats from appropriate cohorts had a sticky-tape ratio of 1, which was expected in healthy animals. After the MCAO procedure, sticky-tape ratio of an animal drops below one and can become zero depending on the extent of stroke-induced somatosensory dysfunction.

Beam-walking test

Beam-walking test often referred to as foot fault test, was used to assess the deficits in coordination and integration of motor movement, especially in the hind limb of rats [36]. Rats from both the cohorts were trained for two to three days to traverse the beam before ischemia induction, and by the end of the training period, all rats learned the task. Beam-walking test was also performed on rats of both the cohorts before ischemia and at regular intervals (1d, 3d, 5d, and 7d) until seven days reperfusion. The beam-walking apparatus consisted of a square, rectangular beam (2 cm x 2 cm cross section and 152 cm long with 110 cm walking distance) connected between two stands. Beam-walking performance of rats was rated as follows: 0 - the rat was not able to stay on the beam; 1 - the rat was able to stay on the beam, but did not move; 2 - the rat tried to traverse the beam, but fell; 3 - the rat traversed the beam with more than 50% foot slips of the affected hind limb; 4 - the rat traversed the beam with more than one-foot slip, but less than 50% foot slips of the affected

hind limb; 5 - the rat traversed the beam with only one-foot slip of the affected hind limb; 6 - the rat traversed the beam without any foot slips of the affected hind limb. Each time a rat is tested, the mean of three trials (at least 15 min resting interval was given between any two trials) was considered for evaluation of beam-walking performance.

Accelerating Rotarod performance test

Accelerating Rotarod performance test was conducted to evaluate the motor coordination of stroke-induced rats that received different treatments. Animals were trained for two to three days before the induction of ischemia, and by the end of the training period, all rats had learned the task. Similar to other tests, this test was also performed before the induction of ischemia and at regular intervals (1d, 3d, 5d, and 7d) until seven days reperfusion. In this test, rats were challenged to stay on the accelerating (0.4 rpm/sec) Rotarod (Rotamex, Columbus instruments) started with an initial speed of 20 rpm. Latency to fall from the accelerating Rotarod was recorded. At each interval, the latency to fall from the accelerating Rotarod for each rat was recorded with a minimum of three trials. The resting time for each rat between any two trials was at least 30 min.

Data collection, exclusion criteria and statistical analysis

Trained research personnel blinded to our treatments performed all the neurological evaluation tests. Animals or the data obtained from the animals were excluded from the study if any of the below-listed criteria were met. (1) Animals that did not meet the set standards of neurological tests during the training period before the induction of ischemia. (2) Animals with the mNSS score of ≤6 when tested after two hours of reperfusion. (3) Neurological data of animals that did not survive until seven days reperfusion. (4) The data of an animal that is extreme/abnormal and not within the data range of the other animals of the same group. Statistical analysis of the data was performed by using Graph Pad Prism software. Quantitative data was tested for normality and equality of variances. The data that were normally distributed within a group at different time points were analyzed by the repeated measures ANOVA followed by the Tukey’s post hoc test. The data between two groups at any given time point that followed a normal distribution with equal variances were analyzed by unpaired t-test. We applied Welch’s correction to the unpaired t-test, if the data were neither normally distributed nor had equal variances. Results are expressed as mean ± SEM. Differences in the values were considered significant at $p<0.05$.

Results

Typical characteristics of exosomes

We collected the exosomes from HUCB-MSCs under standard culture conditions. Immunoblot analysis demonstrated that the typical exosome markers, including CD9 and CD63, were highly expressed in the HUCB-MSCs-derived exosomes that were obtained by the methods described above (Fig. 2A).

Exosomes treatment mitigates the ischemic brain damage

We tested the effect of exosomes treatment on post-stroke brain damage by determining the infarct size and swelling at one-day reperfusion. Representative TTC stained images of

exosomes treated rats euthanized at one-day reperfusion were shown in Fig. 2B. The degree of percent infarct size and swelling in exosomes treated rats were compared with the data of untreated, ischemia-induced rats that we reported earlier [33]. The mean percent infarct size was reduced to 38.65 in exosomes treated rats as compared to 58.9 in untreated, ischemia-induced rats (Fig. 2C). The decrease in infarct size in exosomes treated rats was significant as compared to the untreated, ischemia-induced rats (Fig. 2C; unpaired t-test t=3.485, p=0.0059 exosomes treated vs. untreated, n=6). Further, exosomes treatment reduced the mean percent swelling to 13.09 as compared to 16.32 in untreated, ischemia-induced rats (Fig. 2C). However, the decrease in swelling of the ipsilateral hemisphere in exosomes treated rats as compared to the untreated, ischemia-induced rats was not significant.

Effect of treatment with exosomes on post-stroke body weight changes and mortality

Our experience with MCAO surgeries in a rat model indicates 20–30% mortality after the surgical procedure. Mortality of the animals was recorded throughout the study, and the percent survival rate was calculated. In this study, the post-stroke mortality was within the reported range and the percent survival rate of animals from both the cohorts at seven days reperfusion was the same (Fig. 3A). Rats from vehicle-treated (Fig. 3B; unpaired t-test, t=2.858, p=0.0114 reperfusion day one vs. before ischemia, n=9) and exosomes treated (Fig. 3B; unpaired t-test, t=2.514, p=0.0272 reperfusion day one vs. before ischemia, n=7) cohorts subjected to ischemia showed a significant decrease in post-stroke body weight. There is no difference in the body weight of animals from the exosome treated group compared to the vehicle-treated group either before the induction of ischemia or at one-day reperfusion. Furthermore, the body weight of animals of all the cohorts was recorded at regular intervals until seven days after reperfusion, and the body weight gain was calculated. We did not notice any difference in the percent body weight gain of animals from the exosome treated group as compared to the vehicle-treated group at any of the reperfusion time points tested (Fig. 3C).

Exosome treatment has no effect on the extent of post-stroke injury and recovery

The mNSS test is a composite of motor, sensory, reflex and balance tests. The mNSS value indicates the degree of post-stroke injury. Before the induction of ischemia, rats from both the cohorts have an mNSS score of zero, which was expected for healthy animals. A mean mNSS score of 9.3 at one-day reperfusion in vehicle-treated rats indicated that the degree of post-stroke injury was moderate (Fig. 4). A gradual decrease (Fig. 4; repeated measures ANOVA $p<0.0001$; $F_{(3.32)} = 34.17$; Tukey's post hoc test $p<0.05$ day 3 vs. day 1, $p<0.001$ day 5 vs. day 1, and $p<0.001$ day 7 vs. day 1) of the mean mNSS scores of vehicle-treated rats from 9.3 at one-day reperfusion to 4.1 at seven days reperfusion indicated the natural recovery. The mean mNSS score of animals from exosomes treated group at one-day reperfusion was 7.8, which suggested a moderate post-stroke injury. Although there is a decrease in the mean mNSS score of exosomes treated rats compared to the vehicle-treated rats at one-day reperfusion, the reduction was not significant. Similar to the decreasing trend noticed in vehicle-treated rats, the mean mNSS scores of exosomes treated rats decreased from 7.8 at one-day reperfusion to 4.8 at seven days reperfusion. Except at three days reperfusion (Fig. 4; unpaired t-test t=2.229, p=0.0457 exosomes treated vs. vehicle-treated,

n=5–9), we did not notice any difference between the mean mNSS scores of exosomes and vehicle-treated rats.

Exosomes treatment halts the post-stroke recovery of somatosensory function

The reduction in the mean sticky-tape ratio of vehicle-treated rats from 1 before the induction ischemia to 0.05 at one-day reperfusion indicated severe damage in the somatosensory function of rats (Fig. 5). An increasing trend (Fig. 5; repeated measures ANOVA $p<0.0001$; $F_{(3,32)} = 17.93$; Tukey's post hoc test $p<0.01$ day 3 vs. day 1, $p<0.001$ day 5 vs. day 1, and $p<0.001$ day 7 vs. day 1) in the mean sticky-tape ratio of rats of vehicle-treated group from 0.1 at one-day reperfusion to 0.6 at 7 days reperfusion indicated the natural recovery of somatosensory function. Similar to the vehicle-treated rats, the mean sticky-tape ratio of exosomes treated rats was dropped from 1 before the induction of ischemia to 0.1 at one-day reperfusion. In contrast to the vehicle-treated rats, the mean sticky-tape ratio of exosomes treated rats did not increase from one-day reperfusion to 7 days reperfusion. The prevention of increase in the mean sticky-tape ratio of exosomes treated rats at 5 days reperfusion (Fig. 5; unpaired t-test $t=2.837$, $p=0.0150$ exosomes treated vs. vehicle-treated, n=5–9), and 7 days reperfusion (Fig. 5; unpaired t-test $t=2.871$, $p=0.0141$ exosomes treated vs. vehicle-treated, n=5–9) indicated that the treatment with exosomes halted the natural recovery of post-stroke somatosensory function.

Exosomes treatment after ischemic stroke doesn't improve motor function

In the vehicle-treated animals, the drop noticed in the mean beam walking score and latency to fall from the accelerating Rotarod at one-day reperfusion as compared to their values before the induction of ischemia indicated a severe deficiency in the coordination and integration of motor movement (Fig. 6). The increasing trend in the mean beam walking score (Fig. 6; repeated measures ANOVA $p<0.0001$; $F_{(3, 36)} = 17.1$; Tukey's post hoc test $p<0.01$ day 5 vs. day 1, and $p<0.001$ day 7 vs. day 1) and latency to fall (Fig. 6; repeated measures ANOVA $p<0.0001$; $F_{(3, 28)} = 19.02$; Tukey's post hoc test $p<0.05$ day 3 vs. day 1, $p<0.001$ day 5 vs. day 1, and $p<0.001$ day 7 vs. day 1) of rats of the vehicle-treated group from day 1 to 7 days reperfusion indicated the natural recovery of motor function. There is no difference in the mean beam walking score and latency to fall of the exosomes treated rats compared to the vehicle-treated rats at one-day reperfusion. Except at 7 days reperfusion (Fig. 6; unpaired t-test $t=2.497$, $p=0.0316$ exosomes treated vs. vehicle-treated, n=5–7) in the mean latency to fall, we did not notice any significant difference in the mean beam walking score and latency to fall at the remaining reperfusion time points, although the degree of recovery is less in the exosomes treated rats as compared to the vehicle-treated rats.

Discussion

Despite the advancements in acute stroke care and neurorehabilitation, ischemic stroke remains the leading cause of long-term disability. The therapy for restoring function in patients with residual deficits after stroke is still an unmet clinical need. Several neuroprotective agents deemed successful in preclinical stroke models failed to translate the same in clinical studies. Neuroprotection after ischemic stroke has become the major

translational roadblock. Preclinical studies suggest that cell-based therapies are effective in improving post-stroke functional outcome. Also, several recent clinical trials have reported the cell therapy-mediated improvement in post-stroke outcome [37]. Emerging stroke literature suggests the therapeutic potential of exosomes secreted from cells in various disease models. Treatment with exosomes overcome the limitations associated with cell-based therapies and offer several advantages such as easy entry into the ischemic brain after their administration because of their lipophilicity, less or no immunogenicity and tumorigenicity, and less incidence of occlusion in the microvasculature. In this study, we tested, if the administration of exosomes secreted from HUCB-MSCs under standard culture conditions to stroke-induced rats preserves the neurological function and facilitates the neurological recovery. Previous studies from our laboratory reported the improvement in functional recovery when these HUCB-MSCs were administered to spinal cord injured rats [38]. As expected, the post-stroke seven-day survival rate of all rats was within the reported range associated with MCAO surgeries in rodents and did not differ between groups. A significant decrease in the body weight of rats from both the cohorts at one-day reperfusion indicated the successful induction of ischemia. We expected that exosome treatment immediately after reperfusion prevents post-stroke brain damage and improves the body weight gain of rats at later reperfusion time points. As expected, exosomes treatment attenuated the ischemic brain damage. However, we did not notice any improvement in body weight gain in exosome treated rats as compared to vehicle treated rats until seven days reperfusion, the maximum reperfusion time point tested in the study.

The overall post-stroke neurological function as assessed by the modified neurological severity score, which is a composite of motor, sensory, reflex, and balance tests were graded on a scale of 0 to 18. In this test, one score point was awarded for the inability to perform the test or for the lack of a tested reflex. Therefore the higher score in this test indicates the more severe injury. The degree of damage as assessed by mNSS scores at one-day reperfusion in vehicle-treated rats in this study is similar to the depth of injury reported earlier in a rat two-hour MCAO model [34]. However, in contrast to the reported mean mNSS score at seven-day reperfusion, a more decreased score in vehicle-treated rats suggests a higher speed of natural recovery. The differences in speed of recovery despite the induction of the same depth of ischemic injury could be attributed to the differences in animal strains used in both the studies. Although the treatment with exosomes reduced the degree of damage at all reperfusion time points except day 7, the reduction is statistically significant only at 3-day reperfusion. We do not believe that the difference in outcome as assessed by mNSS test is biologically significant in exosome treated rats compared to the vehicle-treated rats. Our results are in agreement with the earlier report in which the intravenously administered exosomes (100 μg/animal at one-day reperfusion after a 2h MCAO) secreted by MSCs derived from the bone marrow of adult male Wister rats did not show any significant improvement in the mNSS score at seven days reperfusion [30]. Although in their study a significant improvement was reported starting two weeks after treatment in exosome treated rats, we do not expect similar results even if we continue our study beyond seven days because the mNSS scores in exosome treated rats did not show a decreasing trend.

MCAO in rodents leads to both somatosensory dysfunction, and motor impairment. In this study, the post-stroke somatosensory dysfunction, as well as the impairment of motor function and coordination in rats, was assessed by sticky-tape, beam walking, and Rotarod performance tests until seven days reperfusion. Based on the reported literature, we hypothesized that the treatment with exosomes secreted by HUCB-MSCs would prevent post-stroke neurological impairment and facilitate the recovery. In contrast, administration of exosomes to stroke-induced rats immediately after reperfusion, exacerbated the post-stroke somatosensory function and significantly reduced the sticky-tape ratio as compared to vehicle treatment at reperfusion days 5 and 7. We did not notice any difference in the beam walking scores of exosome treated and vehicle-treated rats. Further in exosome treated rats, the post-stroke motor coordination as assessed by the Rotarod test was decreased at reperfusion days 3, 5, and 7 as compared to the vehicle-treated rats and the decrease was significant at seven days reperfusion. Although we expected that the treatment with exosomes secreted from HUCB-MSCs under standard culture conditions might not show any effect on post-stroke somatosensory and motor functions, we did not expect that our treatment would further impair these neurological functions. In contradiction to our expectation, intravenous administration of exosomes secreted from the cells of two independent human bone marrow-derived MSC lines significantly improved the motor coordination of stroke-induced mice [32]. The discrepancy in the outcome could be attributed to the differences in the species, the ischemic duration, the source of MSCs, the frequency of administration, and the dose of exosomes used in these studies.

Based on our research experience of testing HUCB-MSCs in various disease models from the past decade, we noticed that the precise mechanism of how they offer protection mainly depends on the microenvironment they were in. They could behave differently and even capable of producing opposite effects. For example, while HUCB-MSCs treatment induced apoptosis in cancer cells, they inhibited apoptosis when administered to spinal cord-injured or stroke-induced rats [39–41]. Previous studies showed significant differences in microRNA-133b levels of exosomes secreted from rat femur- and tibia marrow-derived MSCs exposed to regular rat brain tissue extracts vs. rat post-ischemic brain tissue extracts [29]. All of these results provided ideas and background for our future studies on exosomes collected from HUCB-MSCs grown in different microenvironments. We hypothesize that the exosomes secreted from HUCB-MSCs at appropriate experimental conditions could serve as promising therapeutic agents for stroke treatment. Therefore, we cultured HUCB-MSCs, subjected the cells to various experimental conditions and collected the exosomes. The experimental conditions to which the cells were exposed, mimicked the *in vivo* situation of ischemic brain cells getting exposed to the administered stem cells. Next, we will focus on *in vivo* experiments in rodent models of ischemic stroke to determine the effect of treatment with these exosomes on post-stroke brain damage and long-term neurological recovery as well as investigate the protection mechanism of microRNAs contained in exosomes secreted by HUCB-MSCs at various microenvironmental conditions. In this study, the molecular constituents of exosomes secreted by HUCB-MSCs under standard culture conditions responsible for the absence or adverse post-stroke somatosensory and motor function outcome is not known. We believe that the treatment with exosomes secreted from HUCB-MSCs on the post-stroke outcome will primarily depend on the molecular constituents of

exosomes, which in turn depends on the cell's microenvironment while secreting the exosomes. Our future studies will investigate the content of exosomes collected under different experimental conditions, and their efficacy in rodent stroke models.

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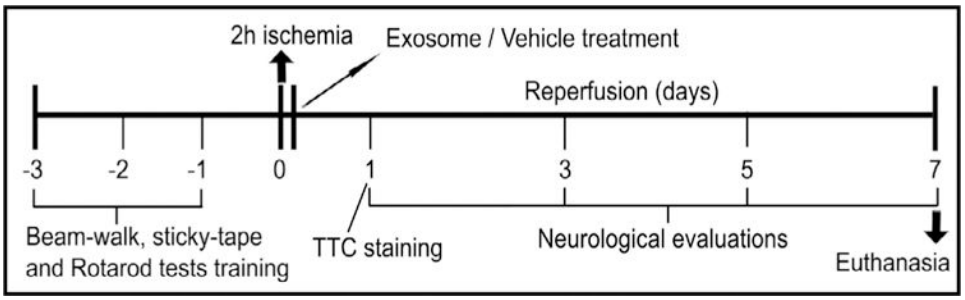

Fig. 1.

Experimental design. Schematic diagram of the experimental design to evaluate post-stroke neurological recovery. Rats were subjected to 2h ischemia followed by 7 days reperfusion with neurological evaluations on reperfusion days 1, 3, 5, and 7, and before ischemia and TTC staining at one-day reperfusion. Appropriate cohorts were administered with either vehicle or exosomes intravenously via tail vein immediately after reperfusion. Rats were trained for beam-walk and Rotarod tests at least for 3 days prior to the induction of ischemia.

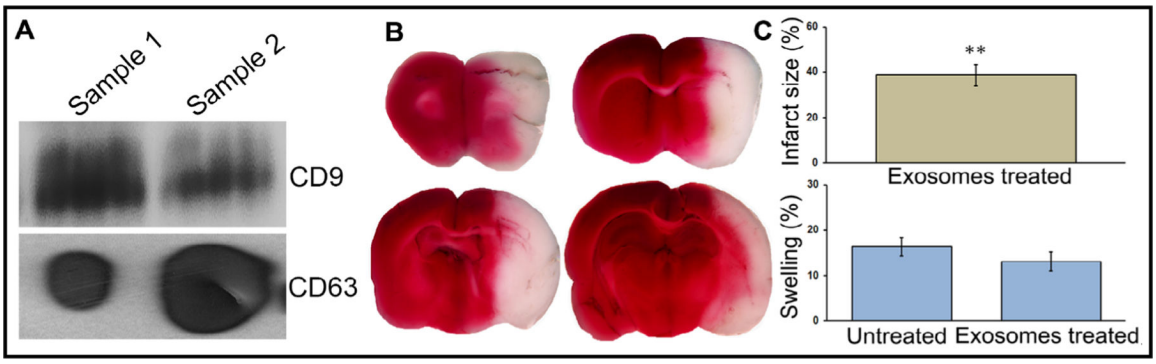

Fig. 2.

Effect of treatment with exosomes on infarct size and swelling. (A) Detection of exosomal markers (CD9 and CD63) expression by immunoblot analysis. (B) Representative TTC stained images of rat coronal brain sections at one-day reperfusion subsequent to a two-hour focal cerebral ischemia in rats. (C) Bar graphs represent the percent infarct size and ipsilateral hemisphere swelling in exosomes and/or untreated rats. Histograms and error bars indicate the mean and the SEM, respectively. n = 6. Quantitative data of infarct size and swelling obtained from exosomes treated rats at one-day reperfusion was compared with our previously published data of untreated, ischemia-induced rats [33]. **p<0.01 vs. untreated ischemia-induced rats euthanized at one-day reperfusion.

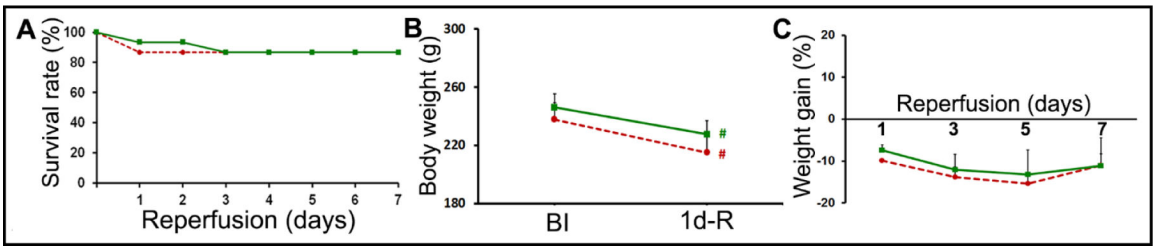

Fig. 3.

Post-stroke mortality and body weight changes. Line graphs represent the percent survival rate (A), the reduction in body weight (B), or percent body weight gain (C) of rats. Dotted, and solid lines represent the data of ischemia-induced animals that were treated with vehicle, and exosomes respectively. BI-before ischemia; R-reperfusion. Error bars indicate SEM. n=15 for mortality assessment and n=7–9 for body weight assessments. $^{\#}p<0.05$ vs. BI.

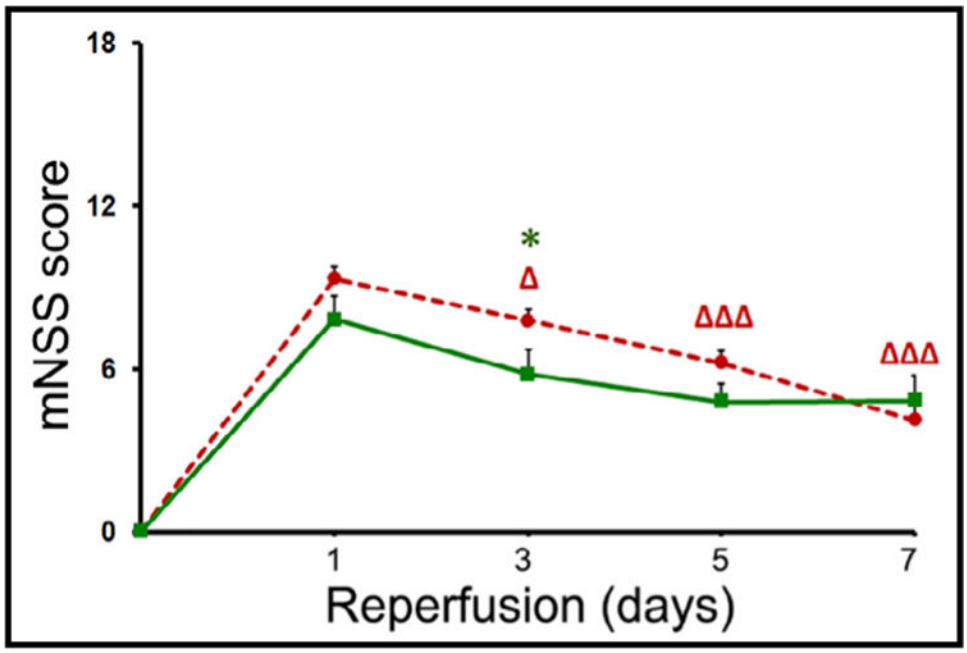

Fig. 4.

Assessment of the severity of post-stroke injury. Severity of the post-stroke injury at one-day reperfusion including the degree of recovery during reperfusion was assessed at regular intervals by the modified neurological severity scores (mNSS) test in rats subjected to 2h ischemia followed by seven days reperfusion. Dotted and solid lines represent the data of ischemia-induced animals that were treated immediately after reperfusion with vehicle and exosomes, respectively. Error bars indicate SEM. n=6–9. $^{\Delta}p<0.05$ vs. 1d reperfusion; $^{\Delta\Delta\Delta}p<0.001$ vs. 1d reperfusion; *$p<0.05$ vs. vehicle treatment at 3d reperfusion.

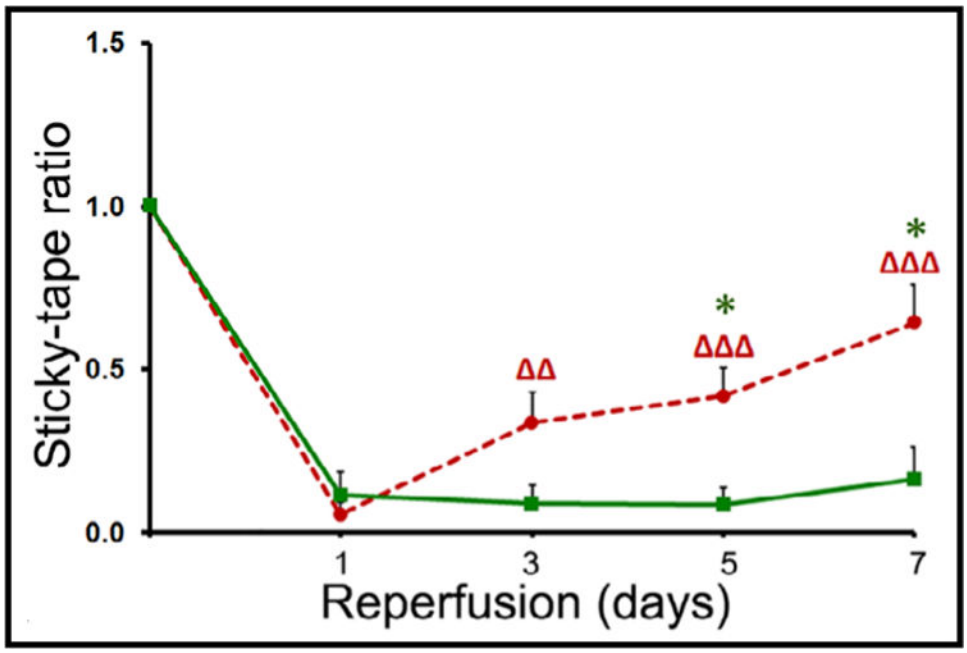

Fig. 5.

Assessment of the post-stroke somatosensory dysfunction. Somatosensory dysfunction at one-day reperfusion including the degree of recovery during reperfusion was assessed at regular intervals by the modified adhesive removal (sticky-tape) test in rats subjected to 2h ischemia followed by seven days reperfusion. Dotted and solid lines represent the data of ischemia-induced animals that were treated immediately after reperfusion with vehicle and exosomes, respectively. Error bars indicate SEM. n=6–9. $^{\Delta\Delta}p<0.01$ vs. 1d reperfusion; $^{\Delta\Delta\Delta}p<0.001$ vs. 1d reperfusion; *$p<0.05$ vs. vehicle treatment at any given time point.

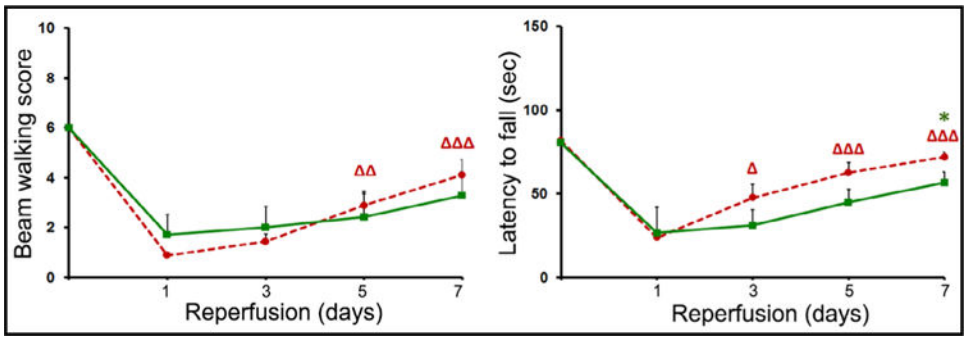

Fig. 6.

Assessment of post-stroke motor coordination and the integration of motor movement. Post-stroke motor coordination and the integration of motor movement at one-day reperfusion including the degree of recovery during reperfusion was assessed at regular intervals by beam walking, and accelerating Rotarod performance tests in rats subjected to 2h ischemia followed by seven days reperfusion. Dotted, and solid lines represent the data of ischemia-induced animals that were treated immediately after reperfusion with vehicle, and exosomes respectively. Error bars indicate SEM. n=5–9. $^{\Delta}p<0.05$ vs. 1d reperfusion; $^{\Delta\Delta}p<0.01$ vs. 1d reperfusion; $^{\Delta\Delta\Delta}p<0.001$ vs. 1d reperfusion; *p<0.05 vs. vehicle treatment at any given time point.