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Mesenchymal stem cell-derived exosomes improve functional recovery in rats after traumatic brain injury: a dose response and therapeutic window study

Yanlu Zhang, MS, MD¹, Yi Zhang, PhD², Michael Chopp, PhD^{2,3}, Zheng Gang Zhang, MD, PhD², Asim Mahmood, MD¹, Ye Xiong, MD, PhD¹

¹Department of Neurosurgery, Henry Ford Hospital, Detroit, MI 48202, USA

²Department of Neurology, Henry Ford Hospital, Detroit, MI 48202, USA

³Department of Physics, Oakland University, Rochester, MI 48309, USA

Abstract

Background.—Mesenchymal stem cell (MSC)-derived exosomes play a critical role in regenerative medicine.

Objective.—This study was to determine the dose- and time-dependent efficacy of exosomes for treatment of traumatic brain injury (TBI).

Methods.—Male rats were subjected to a unilateral moderate cortical contusion. In the dose response study, animals received a single intravenous injection of exosomes (50, 100, 200 µg/rat) or vehicle, with treatment initiated at 1 day after injury. In the therapeutic window study, animals received a single intravenous injection of 100 µg exosomes or vehicle starting at 1, 4, or 7 days after injury. Neurological functional tests were performed weekly after TBI for 5 weeks. Spatial learning was measured on days 31-35 after TBI using the Morris water maze test.

Results.—Compared to the vehicle, regardless of the dose and delay in treatment, exosome treatment significantly improved sensorimotor and cognitive function, reduced hippocampal neuronal cell loss, promoted angiogenesis and neurogenesis, and reduced neuroinflammation. Exosome treatment at 100 µg/rat exhibited a significant therapeutic effect compared to the 50 µg or 200 µg exosome groups. The time dependent exosome treatment data demonstrated that exosome treatment starting at 1 day post TBI provided a significantly greater improvement on functional and histological outcome than exosome treatments at the other two delayed treatments.

Please send all correspondence to: Ye Xiong, MD, PhD, Department of Neurosurgery, Henry Ford Health System, E&R Building, Room # 3096, 2799 West Grand Boulevard, Detroit, MI 48202, Tel: 313-916-4743, Fax: 313-916-9543, yxiong1@hfhs.org.
Author contributions.

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Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Conclusions: These results indicate that exosomes have a wide range of effective doses for treatment of TBI with a therapeutic window of at least 7 days post injury. Exosomes may provide a novel therapeutic intervention in TBI.

Keywords

cell therapy; exosomes; functional outcome; neuroinflammation; neurovascular remodeling; rat; traumatic brain injury

Introduction

Traumatic brain injury (TBI) affects over 10 million people annually leading to mortality and hospitalization worldwide ¹. To date, no effective neuroprotective agent has been identified from clinical TBI trials ². Multipotent mesenchymal stromal cells (MSCs) are self-renewing stem/progenitor cells from bone marrow, adipose tissue, skin, umbilical cord blood, and peripheral blood as well as other organs ³. Previous studies show that a small proportion of transplanted MSCs migrate into injured brain, of which only a small number displayed neural-like markers, indicating functional recovery is not linked to neural differentiation from the transplanted MSCs ^{4, 5}. MSC-induced brain remodeling and functional recovery are associated with their secretion-based paracrine role ^{6, 7}. These therapeutic paracrine effects are likely mediated by MSC generated exosomes ⁸. In contrast to MSCs, nano-sized exosomes can easily pass the blood brain barrier and deliver their cargos including genetic materials, lipids, and proteins to recipient cells ⁹, without vascular obstructive effect and risks of tumor formation ¹⁰.

MSC-derived exosomes significantly improves functional recovery in rats after TBI ¹¹. However, the dose and time-dependent effects of MSC-derived exosomes on functional recovery after TBI remain elusive, which ultimately will determine their clinical utility. The present study was designed to investigate the dose- and time-dependent efficacy of exosomes after TBI in improving cognitive and sensorimotor functional recovery, amplifying neurovascular remodeling, and reducing neuroinflammation after TBI.

Materials and Methods

Animals

Adult (3-month-old) male Wistar rats weighing 339.2 ± 13.6 g (Charles River, Wilmington, MA) were used. All experimental procedures were approved by the Henry Ford Health System Institutional Animal Care and Use Committee. The persons who performed the experiments, collected data, and assessed outcome were blinded throughout the course of the experiments (see Supplemental Materials for details).

Exosome isolation and characterization

Human MSCs were purchased from Theradigm (Bethesda, MD), and expanded, as previously described ¹². MSC-derived exosomes were collected according to the published methods ¹³. Exosome size and particle number were characterized using a NanoSight (Merkel Technologies) instrument. Exosome morphology and exosomal markers (Alix and

Hsp70) were examined by means of transmission electron microscopy and Western blot, respectively. Please see Methods in the Supplemental Materials for details.

Animal model of TBI

A well-established controlled cortical impact (CCI) rat model of TBI was utilized for the present study. Animals were anesthetized intraperitoneally (ip) with ketamine (80 mg/kg) and xylazine (13 mg/kg). Rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ using a feedback-regulated water-heating pad. Rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The second craniotomy was bilaterally symmetrical to the first and performed in the contralateral skull allowing for lateral movement of cortical tissue. The dura mater was kept intact over the cortex.

Cortical injury was delivered by impacting the left parietal cortex (ipsilateral cortex) with a pneumatic piston of a 6-mm-diameter tip at a rate of 4 m/s and 2.5 mm of compression, as described in our previous study¹¹. The cranial defect was not filled after the impact. This moderate TBI induced by CCI targets the parietal cortex, and causes consistent cortical tissue loss (lesion cavity) and significant neuronal cell loss in the ipsilateral hippocampus as well as white matter damage².

Experimental groups

In the dose response experiment, the study animals were randomly divided into 5 groups (n = 8/group): 1) Sham; 2) TBI + Vehicle phosphate-buffered saline (PBS); 3) TBI + 50 μg exosomes/rat (Exo-50); 4) TBI+100 μg exosomes/rat (Exo-100); and 5) TBI + 200 μg exosomes/rat (Exo-200). Exosomes generated from MSCs (50, 100, and 200 μg total protein of exosome precipitate in 0.5 ml PBS/rat or an equal volume of PBS (0.5 ml) were administered intravenously over 5 min via tail vein, starting 1 day after injury. TBI animals treated with PBS served as a treatment control group. Sham animals underwent surgery without injury and treatment.

In the therapeutic window experiment (n=8/group), TBI rats received a single dose of 100 μg total protein of exosome precipitate in 0.5 ml PBS/rat via tail vein, starting at 1, 4, or 7 days post injury, respectively (designated as Group D1, D4, D7). TBI animals receiving 0.5 ml PBS as a control group. Sham animals underwent surgery without injury and treatment.

For labeling proliferating cells, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg) was injected ip into all animals daily for 10 days, starting 1 day after TBI, as described in our previous study¹⁴.

Evaluation of neurological outcome

Modified neurological severity score (mNSS)¹⁵ and foot faults¹⁶ were performed on all rats preinjury and at 1, 7, 14, 21, 28 and 35 days after TBI. To measure spatial learning and memory impairments, the Morris water maze (MWM) test was performed for 5 consecutive days on Days 31-35 after TBI, as described in our previous TBI study¹¹. All neurological

functional tests were performed by observers blinded to the treatment groups. Please see Methods in the Supplemental Materials for details.

Tissue preparation, measurement of lesion volume and immunohistochemistry

On day 35 after TBI, anesthetized rats were sacrificed for collecting brains. Brains were embedded in paraffin and a series of 6- μ m-thick slides were cut. The cortical lesion volume was measured. CD 68-(marker for microglia/macrophages), glial fibrillary acidic protein (GFAP, marker for astrocytes) and neuronal nuclei (NeuN, for neurons)-immunostainings were performed. Please see Methods in our previous study ¹² and the Supplemental Materials for details.

Immunofluorescent staining and cell counting

Newly generated endothelial cells and newborn mature neurons 35 days after TBI were identified by double labeling for BrdU with endothelial barrier antigen (EBA) or neuronal nuclei (NeuN), respectively ¹¹. EBA+ endothelial cells, CD68+ microglia/macrophages, GFAP+ astrocytes, and EBA/BrdU-colabeled cells were counted in the lesion boundary zone (LBZ) and the dentate gyrus (DG) ¹¹. The LBZ is defined as the region of cortical tissue between the lesion cavity and the intact tissue (S1 Fig). For analysis of neurogenesis, we counted NeuN/BrdU-colabeled cells in the subgranular zone and granular cell layer of the DG. The fields of interest were digitized under the light microscope (Nikon, Eclipse 80i) at a magnification of either 200 or 400 using CoolSNAP color camera (Photometrics) interfaced with MetaMorph image analysis system (Molecular Devices). In brief, five fields of view in the LBZ from the epicenter of the injury cavity (bregma -3.3 mm), and 9 fields of view in the ipsilateral DG were counted in each section, as described in detail previously ¹⁷. Please see Methods in the Supplemental Materials for details.

Data analysis

Data are presented as the means with standard deviations. Data were tested for normality. When data were not normally distributed, ranked data or a nonparametric Kruskal-Wallis test was used. Analysis of variance (ANOVA) was used for repeated measurements of spatial performance and sensorimotor function. For cell counting, a one-way ANOVA followed by post hoc Tukey's tests was used to compare the differences between the exosome-treated, PBS-treated and sham groups. Differences were considered significant if the P value was <0.05.

Results

Identification of exosomes from MSC culture medium

Using a qNano nanopore-based exosome detection system, we demonstrated that MSCs generate exosome-enriched particles (147.4 ± 62.6 nm in diameter, with a mode diameter at 122 nm in size, which is consistent with MSC exosomes identified with the transmission electron microscopy (S2 Fig A). Western blot confirmed that exosomes contained high levels of exosome proteins markers Alix and Hsp70 compared to the supernatant (S2 Fig B).

MSC exosome administration significantly enhances spatial learning in rats after TBI

In the dose response study (Fig 1A), compared to the PBS group, post-hoc Tukey's testing demonstrated significantly increased time spent by TBI rats treated with exosomes in the correct quadrant of the MWM test in the 50 µg exosome group at Day 35 ($p < 0.05$), 100 µg exosome group at Day 32-35 ($p < 0.05$) and 200 µg exosome group at Day 34 and 35 ($p < 0.05$). There was a statistically significant improved therapeutic effect with 100 µg than with the 50 µg at Day 34 ($p < 0.05$). There was a significantly reduced latency for TBI rats treated with exosomes in the correct quadrant in the 50 µg exosome group at Day 33-35 (Fig 1B, $p < 0.05$), 100 µg exosome group at Day 32-35 ($p < 0.05$) and 200 µg exosome group at Day 32-35 ($p < 0.05$). There was a statistically significant reduction in latency with 100 µg than 50 µg at Day 32-35 ($p < 0.05$), 100 µg than in 200 µg at Day 33-35 ($p < 0.05$). There was no significant difference in latency between 50 and 200 µg groups at all time points ($p > 0.05$).

In the therapeutic window study (Fig 1C), among the 5 groups, a statistically significant between-group effect on the time spent in the correct quadrant was noted in the MWM test at Day 32 to 35 ($p < 0.001$). Relative to the PBS group, there was a significantly increased time spent in the correct quadrant by TBI rats treated with exosomes in the D1, D4, and D7 exosome treatment groups at Day 33-35 ($p < 0.05$). There was no statistically significant difference in improvement among the 3 exosome groups (D1, D4, D7) from Day 31 to Day 35 ($p > 0.05$). A statistically significant between-group effect on the latency was noted in the MWM test at Day 32 to 35 (Fig 1D, $p < 0.001$). Relative to the PBS group, at Day 32-35, a significantly reduced latency in the correct quadrant was detected in TBI rats treated with exosomes at different time points (D1, D4, D7) ($p < 0.05$). A significant difference in reducing latency was detected between D1 and D4 treatments at Days 33 and 34, between D1 and D4 treatments at Day 35, as well as between D1 and D7 treatment groups at Day 35 ($p < 0.05$), suggesting that the D1 treatment is most effective. There was no significance difference in reducing latency between D4 and D7 treatment groups ($p > 0.05$).

MSC exosome significantly promotes sensorimotor functional recovery in rats after TBI

The mNSS score was identical in the PBS and exosome groups on Day 1 post TBI, indicating neurological functional deficits were comparable in all TBI rats before treatment (Fig 2A, $F_{3,28}=0.24$, $p = 0.87$). A significant reduction in the mNSS score was found over time in the PBS-treated animals starting from Day 7 and up to Day 35 compared to Day 1 post injury ($F_{5, 42} = 76.37$, $p < 0.01$), suggesting a significant spontaneous sensorimotor functional recovery occurring after TBI. In the dose response study (Fig 2A), compared to the PBS treatment, functional recovery was significantly improved in the 3 exosome-treated groups (50, 100, 200 µg/rat) on Days 7-35 after TBI ($p < 0.01$, post-hoc Tukey's test). There was a significant reduction in mNSS in the 100 µg group than in the 50 µg and 200 µg groups at Day 7-35 ($p < 0.05$). There was a significant effect of 200 µg treatment on mNSS compared with the 50 µg treatment group at Day 14-35 ($p < 0.05$). There was no significant difference in footfault tests among TBI groups at Day 1 post injury before assignment of treatments (PBS or exosomes). A significant reduction in the footfault number was found over time in the PBS-treated animals starting from Day 7 and up to Day 35 compared to Day 1 post injury ($F_{5, 42} = 88.95$, $p < 0.01$), suggesting the presence of a significant spontaneous recovery in footfault after TBI. Exosome treatment (50-200 µg/rat) also significantly

reduced the frequency of forelimb footfault occurrence as compared to PBS controls (Fig 2B, at Day 7 to 35, $p < 0.01$). There was a significant reduction in footfault in the 100 μg group than in the 50 μg group at Day 7-28 ($p < 0.05$).

In the therapeutic window study, a statistically significant between-group effect on reducing mNSS was noted at Day 7 to 35 (Fig 2C, $p < 0.001$). Relative to the PBS group, a significantly reduced mNSS was found in TBI rats treated with 100 $\mu\text{g}/\text{rat}$ exosomes (D1, D4, and D7 groups) at Day 21-35 ($p < 0.05$). In addition, early treatments (D1 and D4 groups) significantly reduced mNSS at Day 7 and 14 post injury compared to the PBS group ($p < 0.05$). There was no statistically significant effect on mNSS in the D7 exosome group at Day 14 compared to the PBS groups ($p > 0.05$). The earlier the exosome treatment started, the better effect on reducing mNSS was observed (D1>D4>D7 groups, $p < 0.05$). There was a statistically significant between-group effect on footfaults was noted at Day 7 to 35 ($p < 0.05$). Relative to the PBS group, at Day 14-35, a significantly reduced footfault occurrence was found in TBI rats treated with exosomes at different time points (D1, D4, D7) ($p < 0.05$). There was no significantly improved therapeutic effect on footfault occurrence between D1 and D4 groups ($p > 0.05$). However, the D1 and D4 groups had a significant reduction in footfault occurrence than D7 group at Day 14 ($p < 0.05$).

MSC exosome administration significantly reduces hippocampal neuronal cell loss in rats after TBI

In the dose response study (Fig 3A), compared to the PBS treatment, exosome treatments (50, 100, 200 $\mu\text{g}/\text{rat}$) significantly reduced the NeuN+ neuronal cell loss in the CA3 ($F_{4,35} = 47.00$, $p < 0.05$) and DG region ($F_{4,35} = 328.91$, $p < 0.05$) detected at Day 35 post injury. A statistically significant effect with higher doses of exosomes on reducing neuronal cell loss was found in the DG (100 μg vs 50 μg , 200 μg vs 50 μg , $p < 0.05$). There was no significant effect on neuronal cell number in the CA3 among 3 exosomes doses ($p > 0.05$).

In the window study (Fig 3B), compared to the PBS treatment, exosome treatments (D1, D4, and D7) significantly reduced the NeuN+ neuronal cell loss in the CA3 ($F_{4,35} = 49.05$, $p < 0.05$) and DG region ($F_{4,35} = 235.01$, $p < 0.05$) detected at Day 35 post injury. No statistically significant difference in the effect of exosomes on reducing neuronal cell loss in the DG and CA3 regions among the 3 exosome treatment groups was detected ($p > 0.05$).

MSC exosome administration significantly increases vascular density and angiogenesis in rats after TBI

TBI alone significantly increased the density of vessels measured at Day 35 post injury in the LBZ (Fig 4, $F_{4,35} = 363.55$, $p < 0.001$) and DG ($F_{4,35} = 219.12$, $p < 0.001$) of the ipsilateral hemisphere compared to sham controls. Exosome treatments significantly increased the vascular density in the LBZ and DG compared to the PBS treatment (Fig 4A and 4C, $p < 0.01$). Exosome treatment at 50-200 $\mu\text{g}/\text{rat}$ significantly increased angiogenesis identified by EBA/BrdU+ double labeling for newborn endothelial cells in the LBZ (Fig 4B and 4D, $F_{4,35} = 264.91$, $p < 0.001$) and DG ($F_{4,35} = 300.63$, $p < 0.001$) compared to the PBS treatment. No significant increases in vascular density and angiogenesis in the injured brain were detected between 100 and 200 μg groups ($p > 0.05$). However, the 100 and 200 μg

treatment groups exhibited significantly increased vascular density and angiogenesis compared with the 50 µg group ($p < 0.05$).

In the therapeutic window study (Fig 4E-4H), exosome treatment initiated at Day 1, 4, and 7 post injury at 100 µg/rat significantly increased the vascular density (the number of EBA+ cells) in the LBZ (Fig 4E and 4G, $F_{4,35} = 423.47$, $p < 0.001$) and DG ($F_{4,35} = 385.53$, $p < 0.001$) compared to the PBS treatment. Exosome treatments (Day 1, 4, and 7) significantly increased angiogenesis identified by EBA/BrdU+ cells in the LBZ (Fig 4F and 4H, $F_{4,35} = 332.00$, $p < 0.001$) and DG ($F_{4,35} = 146.57$, $p < 0.001$) compared to the PBS treatment ($p < 0.01$). There was a significant increase in both vascular density and angiogenesis in the LBZ and DG in the D1 group compared to the D4 or D7 groups, $p < 0.05$. There were no significant differences in the vascular density and angiogenesis in the LBZ and DG between the D4 and D7 exosome groups ($p > 0.05$).

MSC exosome administration significantly increases neurogenesis in the dentate gyrus in rats after TBI

TBI alone significantly increased the number of newborn mature neurons in the DG of the ipsilateral hemisphere compared to sham controls measured at Day 35 post injury (Fig 5, $F_{4,35} = 230.50$, $p < 0.001$). Co-localization between BrdU and NeuN was verified using a Zeiss LSM 510 META confocal laser scanning microscope (S3 Fig). However, exosome treatments at doses of 50, 100 and 200 µg/rat significantly increased the number of newborn neurons compared to the PBS control (Fig 5A and 5B, $p < 0.01$). A significant increase in neurogenesis was detected in the 100 and 200 µg groups compared to the 50 µg group ($p < 0.05$). There was no significant difference between the 100 and 200 µg groups ($p > 0.05$).

In the therapeutic window study, exosome treatments at Day 1, 4, and 7 post injury significantly increased the number of newborn neurons compared to the PBS control (Fig 5C and 5D, $F_{4,35} = 209.54$, $p < 0.01$). A significantly increased effect was detected in the D1 group compared to the D4 or D7 group, ($p < 0.05$). There was no significant difference between the D4 and D7 groups ($p > 0.05$).

MSC exosome administration significantly reduces brain inflammation in rats after TBI

TBI alone significantly increased the density of CD68+ macrophages/microglia in the LBZ (Fig 6A and 6C, $F_{4,35} = 1100.10$, $p < 0.01$) and DG (Fig 6B and 6C, $F_{4,35} = 751.50$, $p < 0.01$) of the ipsilateral hemisphere compared to sham controls. Exosome treatments at doses of 50, 100 and 200 µg/rat significantly reduced the CD68+ cell density in the LBZ and DG compared to the PBS treatment (Fig 6C, $p < 0.01$). Compared to the 50 µg group, the 100 and 200 µg groups exhibited significantly reduced CD68+ cell number in the LBZ and DG area ($p < 0.01$). There were no significant differences detected in CD68+ cells between 100 and 200 µg groups ($p > 0.05$). In the therapeutic window study, exosome treatments at Day 1, 4, and 7 post injury significantly reduced the number of CD68+ cells ($F_{4,35} = 826.72$ for cortex and 1023.30 for DG, $p < 0.01$) compared to the PBS controls (Fig 6D-F). The D1 and D4 groups exhibited a significant reduction of CD68+ cells in compared to the D7 group in the LBZ and DG ($p < 0.05$, with ANOVA followed by post-hoc Tukey's test).

TBI also significantly increased the density of GFAP+ astrocytes compared to sham controls (S4 Fig A-C, $p < 0.01$). Exosome treatments (50-200 $\mu\text{g}/\text{rat}$) significantly reduced the GFAP+ astrocyte density in the LBZ and DG compared to the PBS treatment (S4 Fig A-C, $p < 0.01$). The 100 and 200 μg treatment groups exhibited significantly reduced GFAP+ cell number in the injured brain compared to the 50 μg treatment group ($p < 0.05$). There was no significant difference between the 100 and 200 μg treatment groups. There was no significant difference in GFAP+ cells among the D1, D4 and D7 groups (S4 Fig D-F, $p > 0.05$).

Exosome administration does not alter the volume of total brain tissue loss in rats after TBI

No significant differences in the lesion volume were observed 35 days post injury (S5 Fig A-D, $p > 0.05$).

Discussion

The present study demonstrates that intravenous administration of MSC-derived exosomes significantly: 1) improves cognitive and sensorimotor functional recovery in rats after TBI; 2) increases the number of BrdU+/NeuN+ newborn mature neurons in the DG (neurogenesis); 3) increases the number of BrdU+/EBA+ newborn endothelial cells in the LBZ and in the DG (angiogenesis); 4) reduces neuronal cell loss in the CA3 and in the DG (neuroprotection); and 5) reduces the number of CD68+ microglia/macrophages and GFAP+ astrocytes in the LBZ and DG (neuroinflammation). The levels of neuroprotection and neurorestoration are affected in a dose response manner with a greater effect detected at 100 $\mu\text{g}/\text{rat}$ exosomes administered 1 day after injury. In addition, the therapeutic window of exosomes can be extended up to at least 7 days post TBI. These therapeutic effects of exosomes are not confined to a single cell type or region of the injured brain after TBI. Exosomes showed significant effects in both the injured cortex and hippocampus in terms of neuroinflammation and angiogenesis and neurogenesis in the DG.

Among stem cells, MSCs a mixed cell population of stem and progenitor cells, are a promising source of cell-based therapy for TBI because they can be easily isolated from many tissues and expanded in culture from patients without ethical and immune rejection problems^{7, 18}. TBI induces acute and chronic immune response in the brain.⁸ Our recent studies show that intravenous injection of 100 μg total protein of exosomes collected from culture media of approximately 2×10^6 MSCs improves functional recovery in rats with stroke¹⁹ and TBI¹¹. Treatment with exosomes derived from MSCs improve functional recovery and neuroplasticity by reducing long-term neuroinflammation in TBI rats^{11, 12, 20}. In this study, we first determined a dose-response efficacy for this novel mode of exosome treatment for TBI. Our data indicate that a higher dose of exosomes (200 $\mu\text{g}/\text{rat}$) does not provide additional beneficial effects compared to 100 $\mu\text{g}/\text{rat}$ group. However, a lower dose of exosomes (50 $\mu\text{g}/\text{rat}$) provides less beneficial effects than 100 $\mu\text{g}/\text{rat}$. Our dose response study is in line with a recent report showing that intravenous administration of exosomes from adipose tissue derived human mesenchymal stem cells (50 μg , 100 μg and 200 $\mu\text{g}/\text{rat}$, 1 day post injury) improves functional recovery, brain repair, and neural connectivity in rats

with subcortical stroke induced by injecting endothelin-1²¹. Our data suggest that 50-200 µg of exosomes enhance brain repair and recovery in TBI rats, whereas 100 µg of exosomes is the most effective dose when treatment is initiated 1 day post CCI-TBI.

Moderate TBI induced by CCI not only causes cortical tissue loss but also neuronal cell loss in the CA3 region and DG of hippocampus¹¹. The hippocampus is an important brain structure for learning and memory¹¹. Therefore, we analyzed cell loss, angiogenesis and neurogenesis and inflammation in the hippocampus in addition to the LBZ. Emerging preclinical studies indicate that posttraumatic prolonged and progressive neuroinflammation is associated neurodegeneration which may be treatable long after the initiating brain injury²². Neuroinflammation is a key component of the secondary injury cascades following TBI²². In the present study, exosomes significantly suppressed activation of GFAP+ astrocytes and CD68+ microglia/macrophages compared to the PBS control. This anti-inflammatory effect is similar to that of MSC therapy in animal models of stroke^{23, 24} and TBI²⁵. One of main functions for astrocytes and microglia is to monitor and sustain neuronal health by releasing pro and anti-inflammatory cytokines, free radicals, anti-oxidants, and neurotrophic factors²⁶. Activated astrocytes and microglia contribute to both neuronal death and survival in neurodegeneration²⁷ and TBI^{28, 29}. Our data support that suppression of activated microglia/macrophages by exosomes may contribute to increased angiogenesis and neurogenesis, and subsequent improvement in functional recovery after TBI.

To explore the therapeutic window for MSC derived exosome treatment of TBI, onset of exosome treatment was initiated at different times (1 day, 4 days and 7 days post injury). There was a significant time effect on reducing neuroinflammation and neurovascular remodeling in both the injured cortex and hippocampus. Our present data demonstrate that MSC-derived exosomes improve functional recovery in rodents with a wide range of therapeutic window from 1 day to 7 days at a dose of 100 µg after TBI, which is consistent with the therapeutic window of parental MSCs for treatment of TBI rats³⁰⁻³⁵. A recent study in a large animal model of TBI and hemorrhagic shock demonstrates that early (1 hour post injury) treatment with a single dose of MSC-derived exosomes significantly reduces brain swelling and lesion size, lower levels of blood-based TBI biomarkers, and improves blood brain barrier integrity³⁶. However, we note, that we do not exclude the possibility of therapeutic effects with exosome treatments initiated beyond 7 days post TBI.

Exosomes contain complex molecular cargo^{37, 38}. The benefit and potential strength of exosome treatment, as with stem cell therapy, result from targeting multiple injury and repair mechanisms mediated by delivery of exosomal complex cargo to recipient cells. One single microRNA can regulate many genes at posttranscriptional level³⁹. Exosome therapy may transfer miRNA to elicit a multitargeted effect, rather than the traditional, single molecular pathway approach^{8, 40, 41}. The precise mechanisms of exosome therapeutic effects on functional recovery after TBI are not clear. Exosomes can incorporate into recipient parenchymal cells in rats with stroke⁴² and TBI⁸. Intravenous administration of cell-free MSC-generated exosomes improves functional recovery and enhances neurite remodeling, neurogenesis, and angiogenesis after brain injuries in rats^{11, 12, 19}, pigs⁴³, and monkeys⁴⁴. Further studies are warranted to identify the molecular constituents of the exosomes, including specific miRNAs and growth factors that promote angiogenesis and neurogenesis,

and reduce neuroinflammation after TBI. When the specific molecules necessary for a therapeutic effect are identified, selective manipulation of expression of those molecules in the parent MSCs may lead to an enhancement of the therapeutic efficiency of isolated exosomes.

Neurovascular units (NVUs) in the central nervous system consists of endothelial cells, pericytes, neurons, glial cells and extracellular matrix proteins⁴⁵. The generation of new vasculature facilitates highly coupled neurorestorative processes including neurogenesis and synaptogenesis, which in turn lead to improved functional recovery⁴⁵. New neurons are generated from neural stem/progenitor cells occurring in mammals during adulthood and in the pathology of different neurological disorders, and thus neurogenesis may be a potential target area for treatments⁴⁶. Exosome-induced angiogenesis may contribute to motor functional recovery by promoting neurite growth and synaptogenesis in the injured brain¹⁹. Our data in the DG suggest that exosome-induced angiogenesis and neurogenesis may play an important role in improving learning and memory after brain injury, which is consistent with previous studies⁴⁷⁻⁵⁰. In addition to exosomes, MSCs also release other extracellular particles and soluble factors, which may contribute to therapeutic effects underlying MSC therapy^{37, 38}. We do not exclude their effect in MSC-mediated TBI recovery.

In the present study, the cell counting was performed using a profile-based method on a computer monitor to improve visualization and in one focal plane to avoid oversampling^{51, 52}. This method, although suboptimal, provides a meaningful comparison of differences in cell numbers among groups after brain injuries^{51, 52}. Since many investigators seek to determine relative and significant differences in cell numbers between treatment and control groups rather than attempting to estimate absolute cell numbers, it has been argued that in many cases the systematic bias will be canceled out, and thus conventional methods suffice for this task⁵³.

Collecting comparably reliable physical dissector data is very time-consuming requiring considerably more sophisticated equipment than profile counts⁵⁴. Since we are interested in detecting a significant difference in relative cell numbers among groups, the profile counting is the more rational choice. It should be emphasized that when information about absolute cell numbers is required, major biases should be ruled out by calibration or the unbiased stereology method should be employed.

Conclusions

MSC derived exosomes administered intravenously following moderate CCI-TBI in rats improve function recovery with a wide therapeutic window (at least up to 7 days) and offer substantial neuroprotective and neurorestorative effects by reducing neuronal cell loss, neuroinflammation, and amplifying angiogenesis and neurogenesis. Our data and literature suggest that intravenous administration of exosomes may represent a novel therapeutic approach for treatment of acute and subacute TBI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
BrdU	5-bromo-2'-deoxyuridine
CCI	controlled cortical impact
DG	dentate gyrus
EBA	endothelial barrier antigen
FITC	Fluorescein isothiocyanate
MSC	mesenchymal stem cell
GFAP	glial fibrillary acidic protein
ip	intraperitoneally
LBZ	lesion boundary zone
mNSS	modified neurological severity score
MWM	Morris water maze
NTA	nanoparticle tracking analysis
NeuN	neuronal nuclei
NVU	Neurovascular unit
PBS	phosphate-buffered saline
TEM	transmission electron microscopy
TBI	traumatic brain injury

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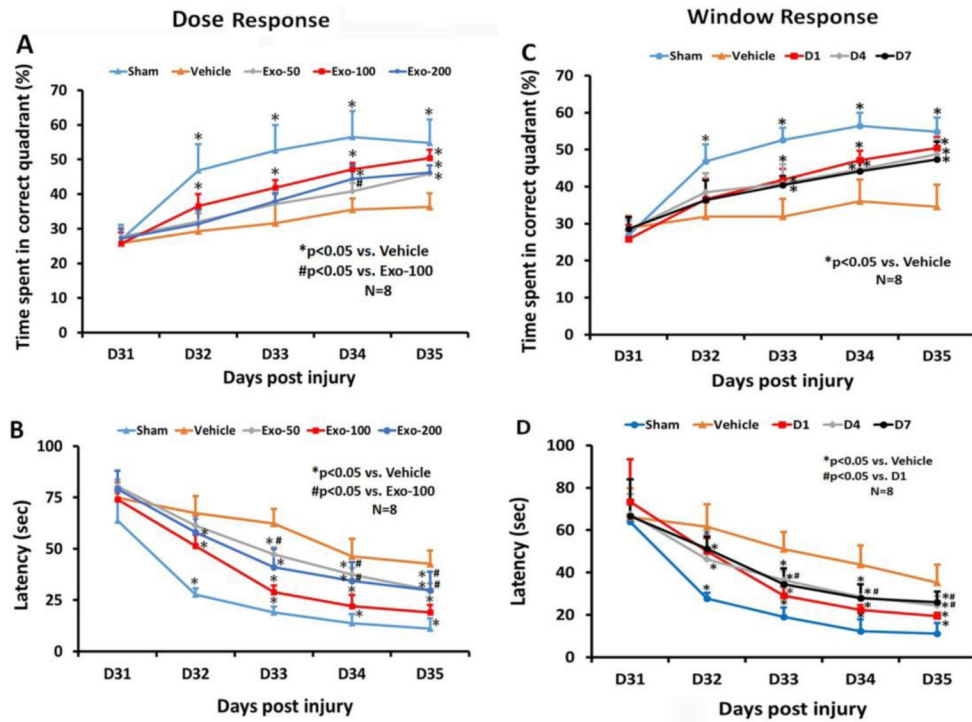


Fig 1. Treatment with exosomes significantly improves spatial learning after TBI. The dose response (**A**, **B**) and window response (**C**, **D**) studies show the percent time spent by animals in the correct quadrant where the hidden platform was located (**A**, **C**), and latency for animals to find the hidden platform (**B**, **D**). Data represent mean \pm SD. N = 8/group.

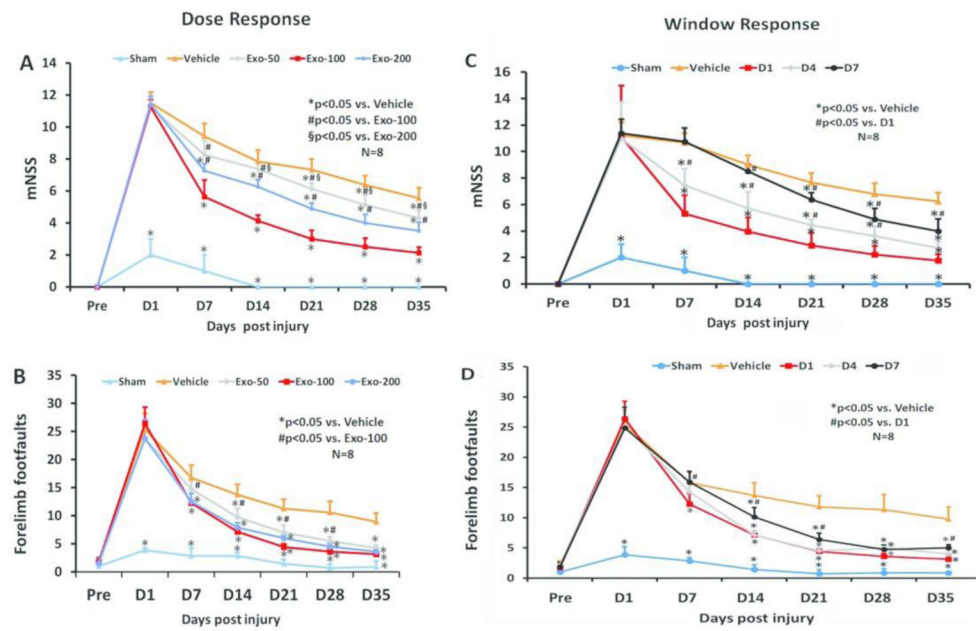


Fig 2. Treatment with exosomes significantly improves sensorimotor function after TBI. The dose response (**A**, **B**) and window response (**C**, **D**) studies show the mNSS score (**A**, **C**) and frequency of foot-fault occurrence (**B**, **D**). Data represent mean \pm SD. N = 8/group.

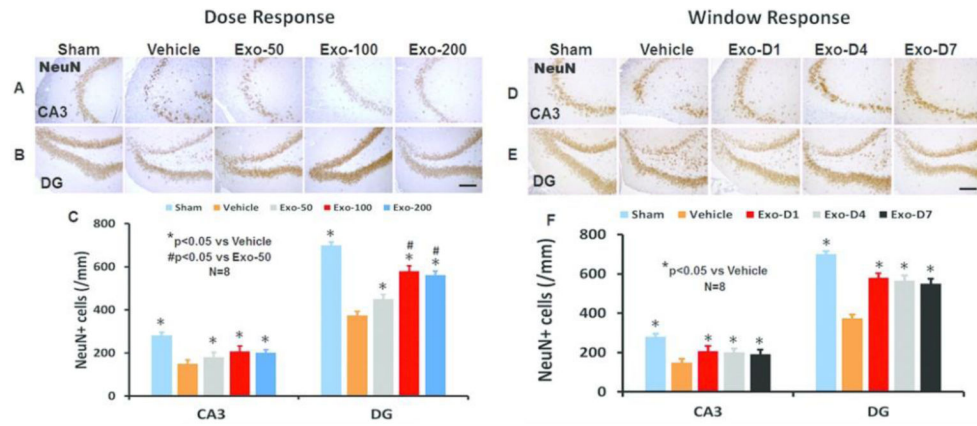
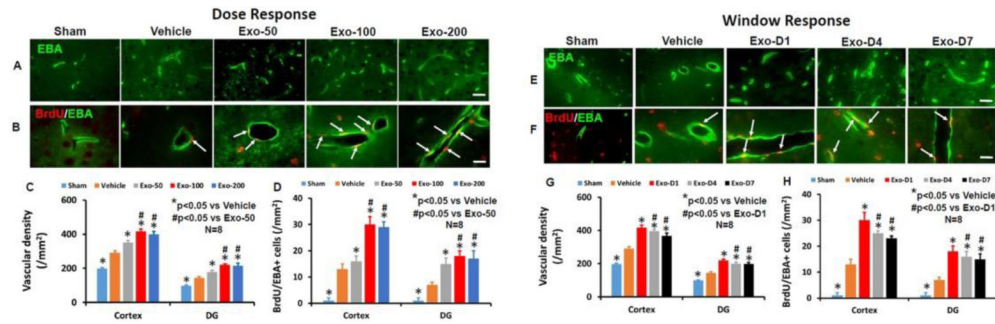


Fig 3. Treatment with exosomes significantly reduces hippocampal neuronal cell loss after TBI. NeuN staining was performed for detection of mature neurons at day 35 after TBI in the CA3 region and dentate gyrus (DG). Representative images show NeuN+ cells in the dose response (**A**, **B**) and window response (**D**, **E**) studies. Scale bars = 100 μ m. Quantitative data shown in bar graphs (**C**, **F**) represent mean \pm SD. N = 8/group.

**Fig 4.**

Treatment with exosomes significantly increases vascular density and angiogenesis after TBI. EBA staining was performed for detection of mature vasculature at day 35 after TBI in the lesion boundary zone (LBZ) and DG. Representative images from injured brain show EBA+ endothelial cells in the dose response (A) and window response (E) studies. Scale bars = 50 μ m. Representative images show BrdU+/EBA+ newly generated endothelial cells in the dose response (B) and window response (F) studies. Scale bars = 25 μ m. Quantitative data on vascular density (C, G) and angiogenesis (D, H) shown in bar graphs represent mean \pm SD. N = 8/group.

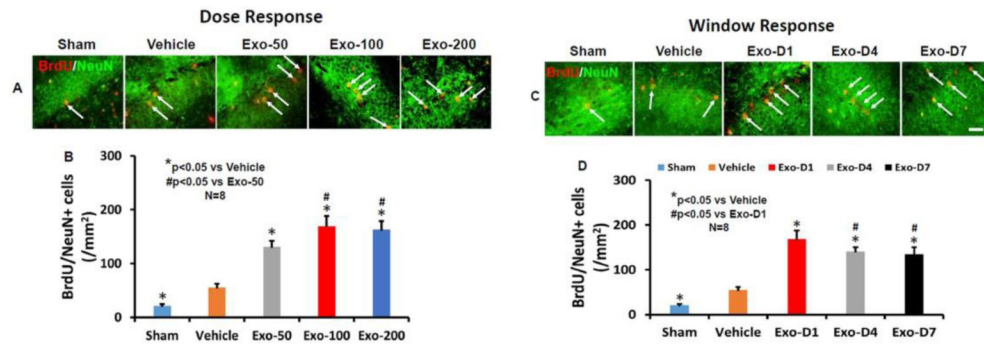
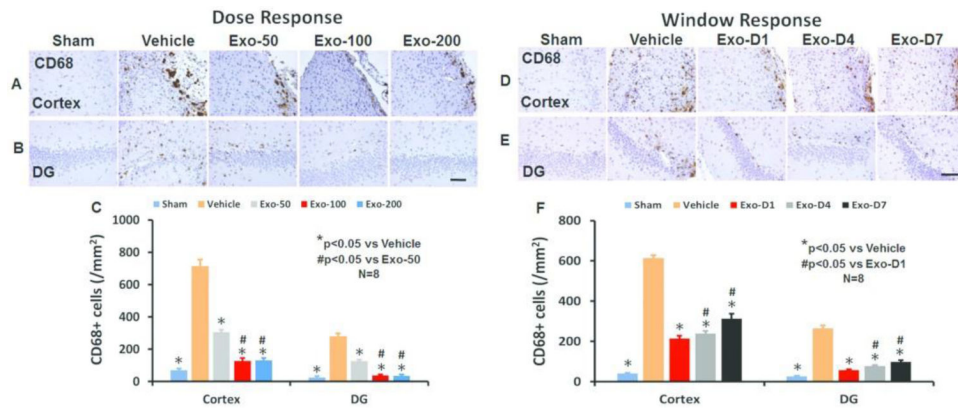


Fig 5. Treatment with exosomes significantly increases neurogenesis 35 days after TBI. Double staining with BrdU (red)/NeuN (green) was performed to identify newborn mature neurons in the DG (yellow arrows). Scale bar = 25 μ m. Representative images show BrdU⁺/NeuN⁺ newly generated neurons in the dose response (A) and window response (C) studies. Scale bars = 25 μ m. Quantitative data shown in bar graphs (B, D) represent mean \pm SD. N = 8/group.

**Fig 6.**

Treatment with exosomes significantly reduces the number of activated CD68+ microglia/macrophages after TBI. CD68 staining was performed to identify activated microglia/macrophages in the dose response (**A** for cortex, **B** for DG) and window response (**D** for cortex, **E** for DG) studies. Scale bar = 100 μ m. Quantitative data shown in bar graphs (**C**, **F**) represent mean \pm SD. N = 8/group.