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Original Article

Intravenous adipose-derived stem cells transplantation ameliorates memory impairment in moderate traumatic brain injury rats via the phosphorylation of extracellular signal-regulated kinase 1/2

Jianguo Wu, Hong Li, Desheng Wang, Desheng Xu, Wei Wang

The Second Hospital of Tianjin Medical University, Tianjin 300211, China

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Abstract: Preliminary studies showed transplantation of adipose-derived stem cells (ADSCs) could actually ameliorate memory impairment in TBI and the mechanisms involved are still to be elucidated. The effect of ADSCs transplantation on memory impairment of rats after TBI were accessed by the Morris water maze (MWM) and the novel object recognition testing (NOR). ADSCs-transplanted rats showed more curiosity versus vehicle-treated control group in NOR. In MWM test, rats in vehicle-treated group required significantly longer time to found the hidden platform than those that were spent by rats in sham and ADSCs transplantation groups. Rats in ADSCs transplantation group had significantly longer latencies to cross the location of the previously hidden platform than rats in vehicle-treated group during the probe trial, and rats in these two groups show significant difference in the number of platform crossing. And expression levels of phosphorylated cAMP response element binding protein (pCREB) and phosphorylated ERK (pERK) in the hippocampus were increased indicated by western blot and immunohistochemical. In conclusion, intravenous ADSCs transplantation could efficiently ameliorate memory impairment in TBI rats. Meanwhile, the learning-associated ERK and CREB activity were predominantly enhanced in the hippocampus.

Keywords: Adipose-derived stem cells, traumatic brain injury, ERK1/2, CREB, BDNF

Introduction

Traumatic brain injury (TBI) is a major public health issue and it can lead to temporary or permanent impairment of memory as well as physical and psychosocial functions [1]. Hippocampal-dependent learning and memory is highly susceptible to TBI, so one prominent memory impairment after TBI is hippocampal-dependent memory loss. However, no treatments can be used in clinic today to improve the memory sequelae of TBI specifically. Developments in stem cell technology in recent years lead to new therapeutic strategies [2, 3]. Mesenchymal stem/stromal cells (MSCs) potentially offer a novel therapy for multiple central nervous system pathologies such as stroke, Parkinson's disease, experimental autoimmune encephalomyelitis, amyotrophic lateral sclerosis, and TBI [4-11], Adipose-derived stem cells (ADSCs) can be extracted from adipose tissue easily, and are capable of expansion *in vitro* with the capacity of differentiating into adipocyte, chondrocyte, myocyte, cardiomyocyte, osteoblastand even neural lineages [12-14]. Due to their wide availability with great proliferation potentials, ADSC cells present as a more clinically feasible source than other adult stem cells for transplantation. They produced beneficial effects in several neurological disorders [15-17]. However, whether transplantation of ADSC cells could actually ameliorate the memory impairment in TBIis is still to be tested.

It has been reported that hippocampal long-term potentiation (LTP, a potential mechanism for learning and memory) is suppressed for days to weeks following moderate TBI [18]. And recent studies indicate that extracellular signal-regulated kinase (Erk), a member of MAP kinases family, plays a critical role in long-lasting neuronal plasticity including LTP and long-term

memory (LTM) [19-21]. Erk cascade activates cAMP response element binding protein (CREB) and other transcription factors involved in the regulation of novel protein synthesis that is required for the stabilization of new memories and modulation of synaptic plasticity [22]. What's more, hippocampal ERK phosphorylation plays a crucial role in spatial working memory [23].

Here we raised a hypothesis that the transplantation of ADSC cells could ameliorate memory impairment. And learning and memory-enhancing effects of ADSC cells transplantation are associated with the phosphorylation of ERK and CREB.

Materials and methods

Animals

A total of 60 adult male Sprague-Dawley (SD) rats and three female ones (250-300 g; purchased from Animal Center of Military Medical Sciences Academy, Beijing, China) were used in this study. Each animal was housed in an independent cage with controlled temperature (22 ± 1°C) and humidity (50% relative) as well as a 12-h light/dark cycle. Food and water was available ad libitum throughout the experiment. The rats were divided into sham, FPI + vehicle and FPI + ADSCs transplantation groups, with 20 rats in each group, Animal handle and procedures were in accordance with the National Institutes of Health guidelines and approved by the Tianjin Medical University Animal Care and Use Committees. The number of animals used in these experiments was minimized.

Isolation of adipose-derived stem cell

Isolation and culture of ADSC cells were performed as previously described [24]. Perirenal and epididymal adipose tissuesof three male SD rats was collected and washed with sterile cold Hank's balanced salt solution (HBSS, Gibco, Gaithersburg, MD, USA) containing 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA); the tissue was minced and then digested using collagenase type I (Gibco) at 37°C for 24 h. Filtering with a 70-um cell strainer (BD Biosciences, San Jose, CA, USA), flow-through was centrifuged to separate stromal vascular fraction from floating adipocytes. Precipitation was then re-suspended and plated onto a cell culture dish (Corning, Corning, NY, USA) at a density of 10^5 cells/ml in fresh Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Cell cultures were maintained in a standard humidified incubator. Cells were collected at the third passage and analyzed by flow cytometry, ADSC cells treated with lipogenic medium for 3 weeks were stained with oil red O to mark neutral lipids in the cytoplasm, and treated with osteogenic medium for 3 weeks were stained with Alizarin red to mark mineralized matrix. Cells from the third passage were collected to be used for experiment.

Surgical procedure

A fluid percussion injury (FPI) device (University of Virginia) was used to administer a unilateral brain injury, as described previously [25, 26]. Rats were initially anesthetized with 4% isoflurane for anesthesia induction and later to 2% isoflurane for maintenance. Strict sterile technique was kept during surgical procedures. Rats were mounted in a stereotaxic frame (ZS Dichuang Lab, Beijing, China), a scalp incision was made along the mid-line, and a 3.0-mm-diameter craniotomy was performed with a trephine on the right parietal bone (centered at 3.0 mm behind of the bregma and 3.0 mm right lateral to the sagittal suture). A modified Luer-lock connector 2.4 mm in inner diameter was secured to the craniotomy with dental acrylic. Moderate TBI (1.8 ± 0.02 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a FPI device. The animal was removed from the device immediately after injury, and anesthesia was maintained for 5 min, while the connector was removed and the incision sutured. Each animal was ventilated on room air without isoflurane until spontaneous breathing resumed, Sham rats were anesthetized and received craniotomies except those rats with no pressure pulse delivered.

Intravenous transplantation

Rats were anesthetized with chloral hydrate at 35 mg/100 g body weight intraperitoneally, and 4×10^6 ADSC cells were suspended in 1 ml of PBS. Cells in PBS or PBS alone were injected

via the tail vein at the 1st day, 3rd day and 7th day after TBI.

Behavioral experiments

Morris water maze (MWM) and Novel Object Recognition testing (NOR) were performed to examine the spatial learning/memory function of TBI rats transplanted with PBS or ADSC cells (n = 6 in each group).

MWM test was performed as previously described [27-29]. The maze consists of a circular pool (150 cm diameter, 60 cm depth, Beijing Xintiandi Technologies, Beijing, China) with black interior filled with water at the depth of 28 cm. Water temperature was maintained at 24 ± 2°C. A transparent polyvinyl chloride escapes platform (12 cm diameter), which approximately 1 cm below the water surface. Signs of distinct colors were pasted on each wall of the four quadrants to serve as visual position clues. A computerized tracking system (Noldus, Wageningen, Netherlands) was used to track and record animal movement and swimming pattern in the maze. At the 16th day after transplantation, the animals were trained with four trials per day for 5 consecutive days, with different starting locations between trials. Each animal was placed in water against the wall at the starting point, and it was given 60 s to swim to locate the hidden platform beneath the water surface. If the platform was not located after 60 s, the rats would be gently guided to the location and was allowed 10 s on the platform. Escape latency was measured as the time rats took to locate the platform. In the last trial, the navigation paths of the rats from the starting point to the platform were recorded. The probe test was performed on day 5, 6 hours after the navigation path recording. The platform was taken away from the pool, and each animal was allowed to swim freely in the water for 120 s; the times of crossing the exact location where the platform used to be placed was recorded.

The NOR task was carried out according to the previously described procedure [30, 31]. The experiments were performed in a square open-field arena (45 × 45 × 45 cm) made of dark gray polyvinyl chloride (Beijing Xintiandi Technologies). Two iron parallelepipeds (3 × 3 × 5 cm) were put in the back left and right corners of the apparatus. Rat was allowed (one at a time) to explore them for 10 min, and then the rats was returned to its home cage. After a 1 h retention interval, the rat was placed back at the mid-point of the wall opposite the sample objects. One circular familiar object with similar texture and size were placed. Each rat was allowed to explore for 5 min. Exploration occurred when the animal sniffed or touched the objects with nose and/or forepaws. The times of exploration were manually recorded. The recognition index (RI) was evaluated by the ratio of TN/(TF + TN), where TF represented times of exploring the familiar object and TN represented times of interacting with the novel one.

Western blot analysis

Rats were killed at the 7th days, 14th days and 21st days post-transplantation, Hippocampal tissues (n = 5 per group) were isolated from the rats and homogenized in ice-cold lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich). The lysate was centrifuged at 18,000 ×g at 4°C for 15 min. The supernatant was estimated by method of Bradford. The supernatant (25 μg protein) was subjected to 8-10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF 0.45 μm; Millipore, Bedford, MA, USA) PVDF were blocked in a TBS-T buffer, containing 5% BSA, 0.1% Tween 20, then incubated with anti-pERK (Cell signaling, CA, USA), anti-ERK (Santa Cruz), anti-pCREB (Upstate, Lake Placid, NY, USA), anti-CREB (Santa Cruz), or anti-BDNF antibodies (Santa Cruz) (1:1000 dilution) at 4°C overnight. On the second day, PVDF were subsequently washed 3 times with TBS-T and then with horseradish peroxidase conjugated secondary antibodies (1:2000 dilution, Abcam, Cambridge, UK) at room temperature for 1 hour. Blots were detected using an enhanced chemiluminescence (ECL)-detection system (Amersham Pharmacia Biotech, Sunnyvale, CA, USA). The experiment was duplicated three times.

Immunohistochemistry

Following the completion of the behavioral tasks. Those rats (n = 5 per group) were killed and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed and post-fixed in phosphate buffer containing 4% paraformaldehyde overnight. Then it was im-

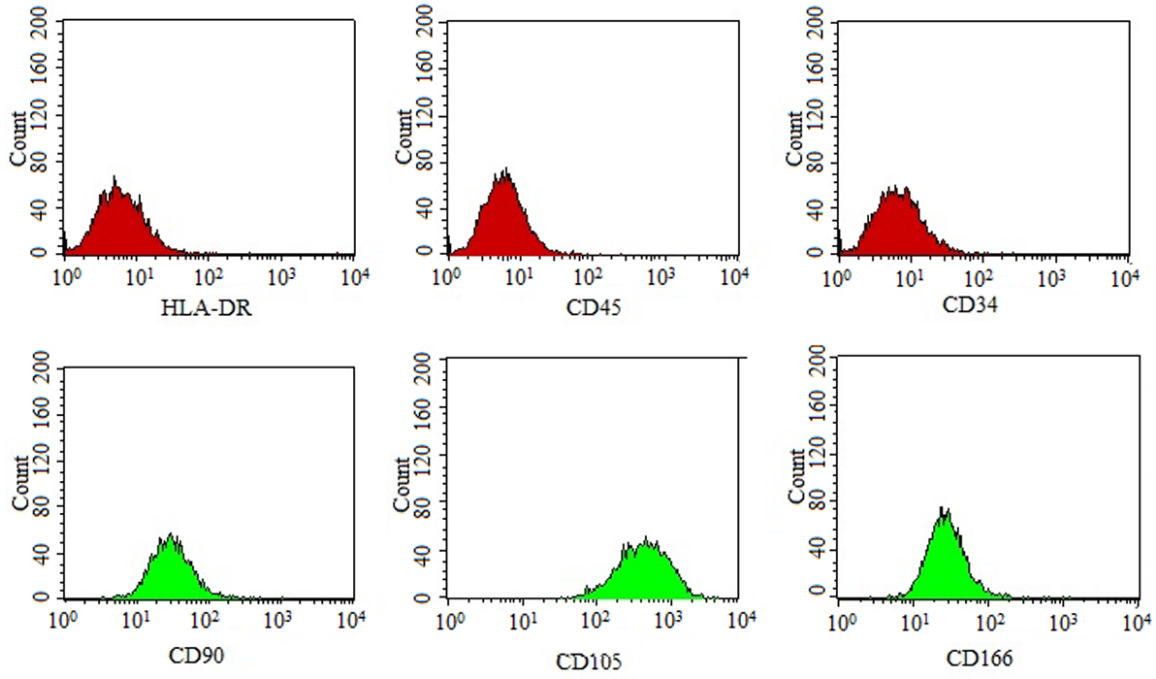

Figure 1. Flow cytometry characterization of adipose-derived mesenchymal (ADSC) cells before administration. ADSC cells were analyzed before administration. These cells were positive for stem cell markers $CD90^+$ (99.02 ± 0.35%), $CD105^+$ (98.89 ± 0.64%), and $CD166^+$ (99.01 ± 0.84%), and negative for $CD44^+$ (1.01 ± 0.34%), $CD34^+$ (2.43 ± 0.21%) and $HLA\text{-}DR^+$ (1.34 ± 0.46%). These results indicated that these cells maintain a typical ADSC phenotype.

mersed in 30% sucrose solution and stored at 4°C, and were sectioned into 40-μm thick slices using a cryostat, Free-floating sections were incubated overnight in polyclonal anti-BDNF antibody (1:1000 dilution), anti-pCREB antibody (1:1000 dilution) or anti-pERK antibody (1:1000 dilution) in PBS containing 3% Triton X-100, 2% BSA and 1.5% normal horse serum. The sections were then incubated with biotinylated secondary antibody (1:200 dilution) for 90 min or avidin-biotin-peroxidase complex (1:100 dilution) at room temperature for 1 h. The sections were then reacted with 0.02% 3,3'-diaminobenzidine and 0.01% H_2O_2 for about 3 min. Finally, they were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series and cleared in xylene. After each incubation step mentioned earlier, the sections were washed three times with PBS.

Quantitative immunostaining and Western blotting

Cell counts in the hippocampal CA1 layer were determined using computerized image analysis system (Leica Microsystems AG, Wetzlar, Germany) in six sections per rat by one person who was unaware of the treatments given. Film densitometry analysis of Western blots was performed using a Quantity One Image Analysis System (version 4.6.3, Bio-Rad Laboratories, Hercules, CA, USA). Levels of phosphorylated ERK and CREB expression were determined by calculating the ratio of phosphoprotein density to total protein density in same membranes. BDNF expression levels were normalized by actin levels in same membranes.

Statistical analysis

Data were presented as mean ± SEM. Analysis of variance (ANOVA) with Bonferroni correction was applied to perform statistical comparisons. Two-way repeated measures ANOVA with Student-Newman-Keuls multiple comparison test was also carried out with Prizm software for water maze spatial learning test to investigate statistically significant progressive decreases in latency to reach the hidden platform. *P* values of < 0.05 were considered as statistically significant.

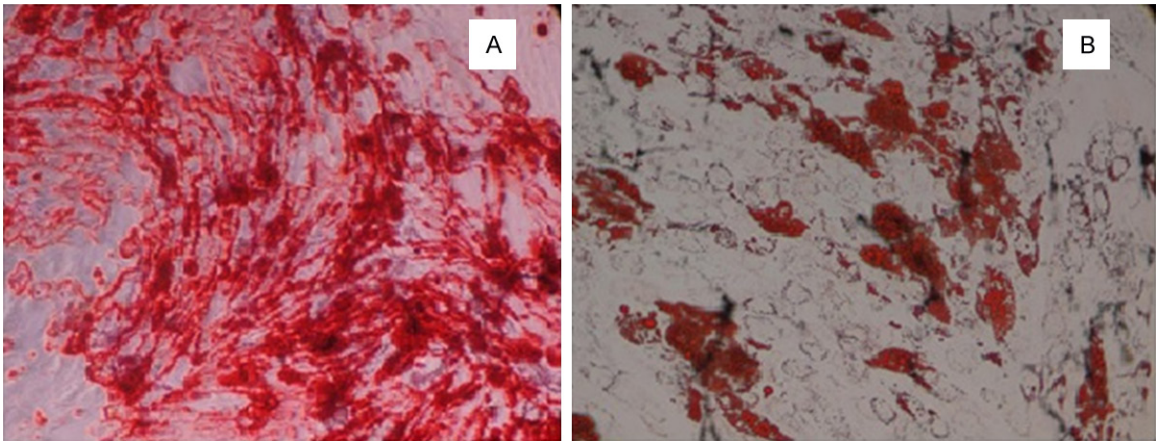

Figure 2. ADSC cells treated with lipogenic medium for 3 weeks were stained with oil red O to mark neutral lipids in the cytoplasm (A); ADSC cells treated with osteogenic medium for 3 weeks were stained with Alizarin red to mark mineralized matrix (B). Magnification: 400 ×.

Result

Cell surface markers were determined

ADSC cells treated with lipogenic medium for 3 weeks were stained with oil red O to mark neutral lipids in the cytoplasm (**Figure 2A**); ADSC cells treated with osteogenic medium for 3 weeks were stained with Alizarin red to mark mineralized matrix (**Figure 2B**).

Flow cytometry demonstrated that these cells were positive for stem cell markers $CD90^+$ (99.02 ± 0.35%), $CD105^+$ (98.89 ± 0.64%), $CD166^+$ (99.01 ± 0.84%), $CD44^+$ (1.01 ± 0.34%), $CD34^+$ (2.43 ± 0.21%), HLA-DR^+ (1.34 ± 0.46%) (**Figure 1**). These results indicated that these cells maintain a typical ADSC cells phenotype.

ADSC cells transplantation alleviates memory impairment in TBI rats

Vehicle-treated group required significantly longer time to find the hidden platform than those spent by rats in sham and ADSC cells transplantation groups (**Figure 3A**). During probe trial, rats in the ADSC cells transplantation group demonstrate significantly longer latencies to cross the location of the previously hidden platform than rats in Vehicle-treated group did. Representative probe trials from each group are shown in **Figure 3B**. Consistent with the longer latencies recorded during the probe trials, analysis of the number of platform crossings also revealed significant differences between Vehicle-treated group and ADSC cells transplantation group (ADSC cells transplantation = 4.33 ± 0.72 crossings, Vehicle = 3.06 ± 1.16 crossings, $P < 0.05$) (**Figure 3C**). However, the average swimming speed was not significantly deferent between rats from these two different groups.

TBI rats treated with PBS exhibited no discrimination between the novel and familiar objects, while ADSC cells-transplanted rats showed more curiosity about the novel one (**Figure 3D**). These results demonstrated ADSC cells transplantation could alleviate memory impairment in TBI rats.

ADSC cells transplantation increases Erk phosphorylation in the hippocampus of moderate TBI rats

Total Erk levels do not change over time following injury in all three groups (data not shown). pERK protein levels in the ADSC cells-transplanted rats' hippocampus were found to significantly increased compared with those in vehicle-treated control rats ($F = 19.497$, $P = 0.017$, **Figure 4A**, **4B**]. Furthermore, these results were supported by immunohistochemical findings [$F = 14.095$, $P = 0.008$, **Figure 5A**, **5D**]. And there is no different between sham-operated and vehicle-treated rats.

ADSC cell transplantation increases CREB phosphorylation in the hippocampus of moderate TBI rats

Total CREB levels do not change over time following injury in three groups (data not shown).

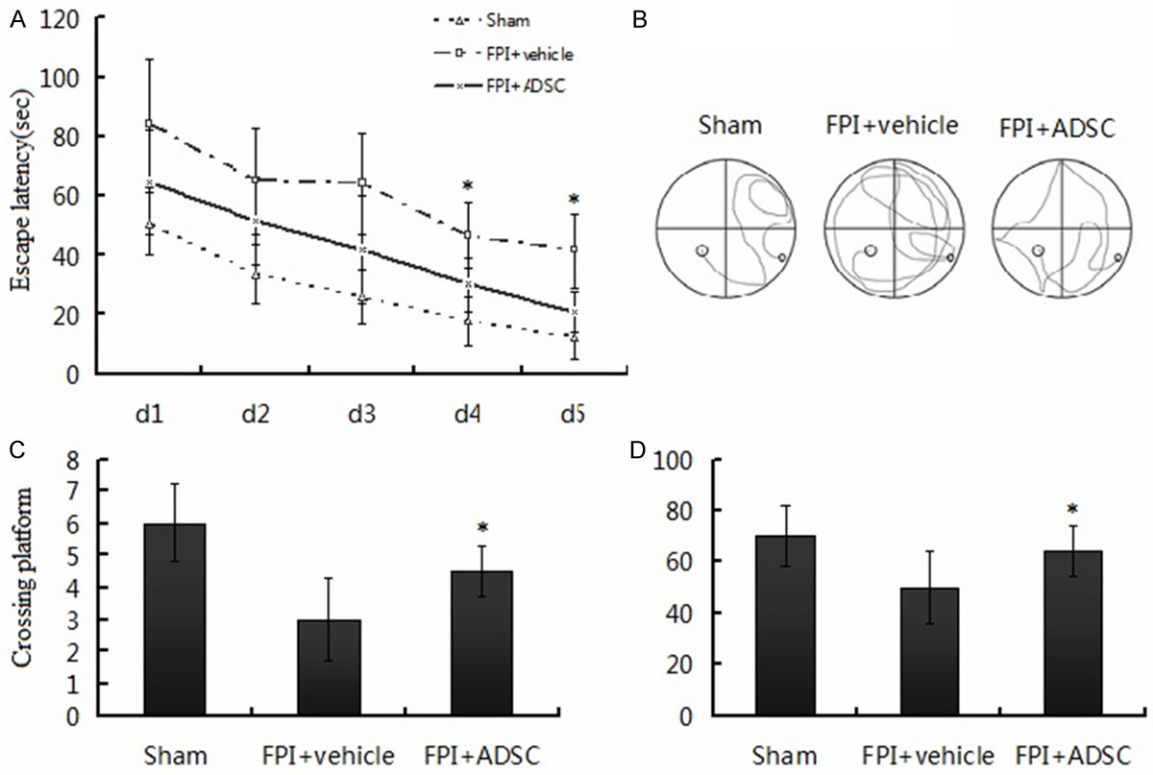

Figure 3. ADSC transplantation alleviates cognitive impairment of TBI rats. A: Escape latencies of LFPI + vehicle or LFPI + ADSC TBI rats and Sham litter mates in Morris water maze task (n = 5). B: Representative navigation path recording on Day 5 of the behavioral performance test. C: Single probe test of spatial learning assessment on the final day (n = 5). D: Learning/memory capability evaluation of vehicle or ADSCs and the Sham with the novel object recognition task (n = 5). Data shown are presented as mean ± SD. *$P < 0.05$, compared with vehicle-treated rats.

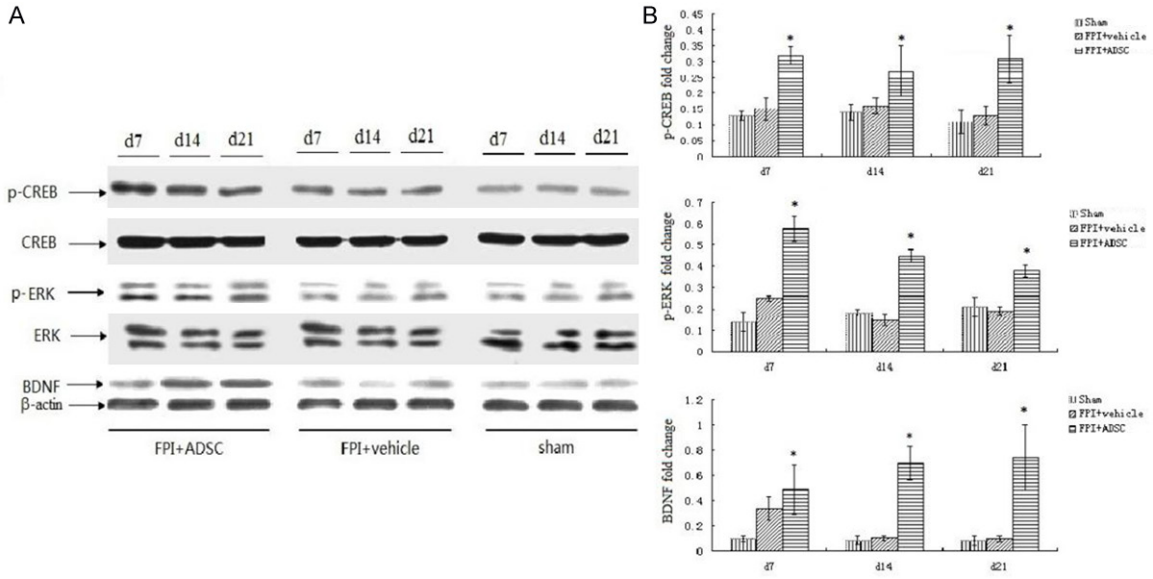

Figure 4. Intravenous ADSC transplantation increases brain-derived neurotrophic factor (BDNF), phosphorylated cAMP response element binding protein (pCREB) and phosphorylated extracellular signal-regulated kinase (pERK) protein levels in the hippocampus. A, B: BDNF, pCREB and pERK immunoreactivities as determined by Western blotting and densitometry. In graph B, the BDNF scale shows normalized ratios versus β-actin, whereas the other normalized ratios were calculated versus their respective total levels. Data represent means ± SEM (n = 5 per group). *$P < 0.05$ versus respective vehicle controls.

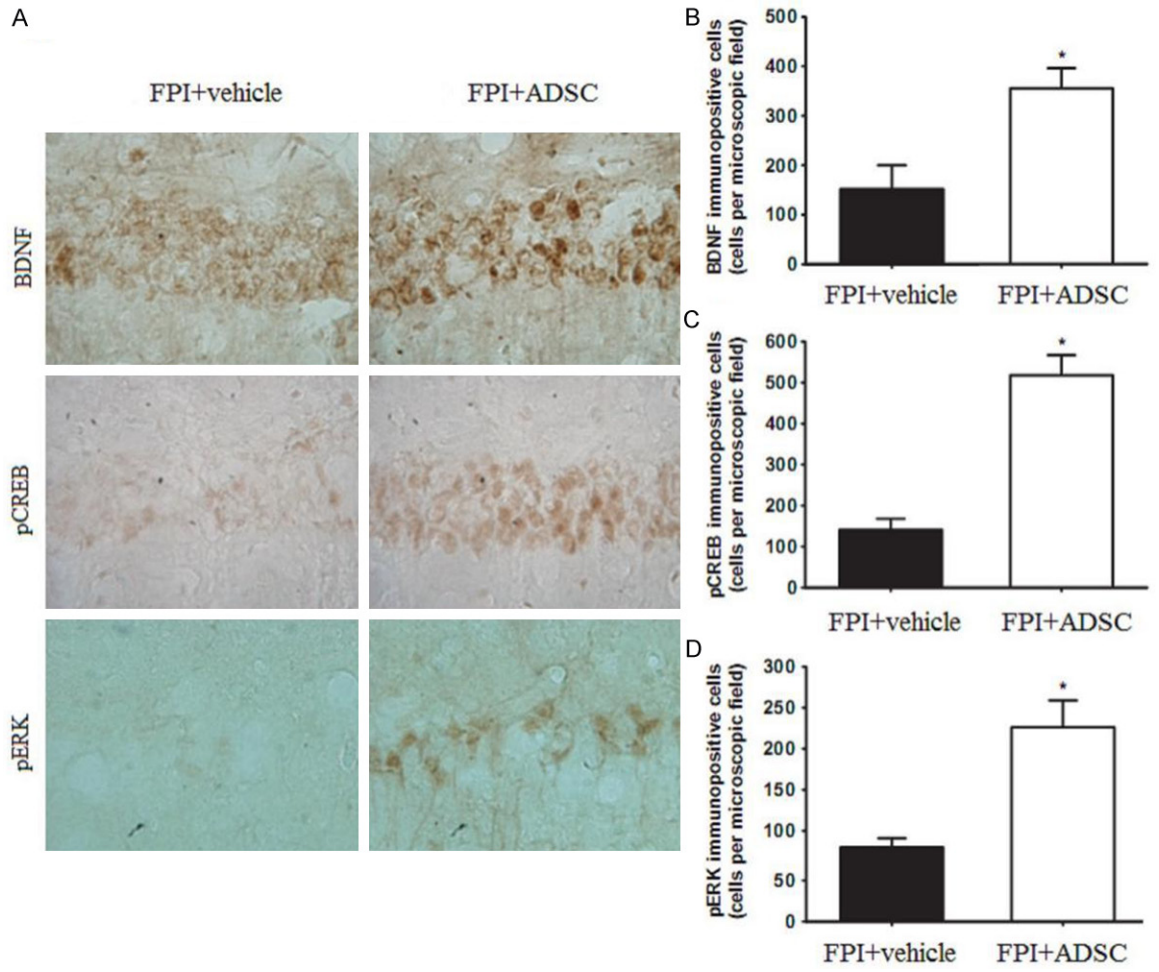

Figure 5. Photomicrographs of brain-derived neurotrophic factor (BDNF), phosphorylated cAMP response element binding protein (pCREB) and phosphorylated extracellular signal-regulated kinase (pERK)-positive cells in the hippocampal CA1 region (A). Summary data of the numbers of BDNF-(B), pCREB-(C) and pERK-(D) positive cells in CA1. Data represent means ± SEM for six determinations in each region for five animals. *$P < 0.05$, compared with sham controls. Magnification: 1000.

pCREB protein levels in the ADSC cells-transplanted rats' hippocampus increase versus that in rats from vehicle-treated controls [F = 17.221, P = 0.006, **Figure 4A**, **4B**], and the immunohistochemical analysis results supported this finding [F = 31.336, P = 0.009, **Figure 5A**, **5C**]. On the other hand, levels of BDNF, a target protein of pCREB, also up-regulated indicated by Western blotting [F = 19.347, P = 0.006, **Figure 4A**, **4B**] or by immunostaining [F = 21.791, P = 0.024, **Figure 5A**, **5B**].

Discussion

Previous preclinical research demonstrated that transplanted mesenchymal stromal cells (MSCs) could migrate to the site of injury and develop neuronal cell markers indicating their differentiation into neurons [neuronal nuclei (NeuN)] and astrocytes [glial fibrillary acidic protein (GFAP)] [32]. However, the frequency and clinical significance of progenitor cell "trans-differentiation" is still debatable. Coyne et al [33] showed that MSCs labeled with BrdU transferred their markers to replicating neurons and gave the deceitful impression that MSCs were expressing these proteins when double labels were used. In addition, hematopoietic stem cells (HSCs) implanted into a spinal cord injury site [34] failed to trans-differentiate into neurons but into macrophages and microglia. Furthermore, Hunt et al [35] demonstrated trans-differentiation induced by collagen deposition and axonal injury after the

implantation of MSCs into demyelinated spinal cord was denied. Although many studies pointed towards trans-differentiation as a potential mechanism for cognitive improvement, many preclinical research certified that this is an unlikely the pathway towards functional benefit. Therefore, we believed intravenous ADSC transplantation did not differentiate into functional neurons in juried area to restore the cognitive function instead they secret various cytokines, inflammation mediators to exert its beneficial effect.

Memory loss (both anterograde and retrograde) is a hallmark of moderate TBI [36]. Following TBI, The hippocampal long-term potentiation is also suppressed in rat [18]. Meanwhile, several protein kinase signaling pathways were modulates in the hippocampus by TBI, such as the mitogen-activated protein kinase (MAPK) signaling cascade. ERK and CREB are critical for hippocampal-dependent memory formation. ERK1/2, which is important downstream signaling mediators of several receptors, was implicated in learning and memory [37]. Previous observations suggest that hippocampal ERK phosphorylation plays a crucial role in spatial working memory [38]. The increased Erk activity preserve neuronal function while inhibiting ERK phosphorylation causes memory impairments. Its blockade exacerbates the loss of both pre- and post-traumatic memories. CREB, a transcription factor, is also required for hippocampus-dependent LTM formation [39]. Its phosphorylation depends on the activation of ERKs, PKA or CaMKII. Furthermore, phosphorylation of CREB activates BDNF or c-fos expression, which are targets of CREB [40].

In the present study, following parasagittal fluid percussion brain injury in the rat, notably memory impairment was observed in the vehicle-treated rats compared to sham-operated rats. And ADSC cells transplantation can increase the phosphorylation of ERK and CREB in the hippocampus which was not seen in rats that were vehicle-treated. Also, we found that ADSC cells transplantation significantly enhanced learning and memory indicated by NOR task results. They also ameliorated spatial learning and memory impairment induced by moderate TBI shown by Morris water maze tasks.

In conclusion, our study demonstrates that intravenous ADSC cells transplantation could efficiently ameliorate memory impairment in TBI rats. Meanwhile, The learning-associated ERK and CREB activation predominantly enhanced in the hippocampus following ADSC cells transplantation. We proposed that ADSC cells transplantation exerts memory improvement through the activation of ERK signaling in the hippocampus. But the detailed molecular mechanism is to be elucidated.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianguo Wu, The Second Hospital of Tianjin Medical University, 23 Pingjiang Road, Hexi District, Tianjin 300211, China. Tel: +86-18622818538; E-mail: wood2008cn@sina.com

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