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Original Paper

Bone Marrow Mesenchymal Stem Cell Transplantation Increases GAP-43 Expression via ERK1/2 and PI3K/Akt Pathways in Intracerebral Hemorrhage

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Key Words
Intracerebral hemorrhage • Axonal regeneration • Stem cells • Neuroprotection

Abstract
Background/Aims: Intracerebral hemorrhage (ICH) occurs in hypertensive patients and results in high rates of mortality and disability. This study determined whether bone marrow mesenchymal stem cell (BMSC) transplantation affects axonal regeneration and examined the underlying mechanisms after the administration of PD98059 (p-ERK1/2 inhibitor) or/and LY294002 (PI3K inhibitor). The hypothesis that was intended to be tested was that BMSC transplantation regulates the expression of growth-associated protein-43 (GAP-43) via the ERK1/2 and PI3K/Akt signaling pathways. ***Methods:*** Seventy-five male rats (250–280 g) were subjected to intracerebral blood injection and then randomly received a vehicle, BMSCs, PD98059 or LY294002 treatment. Neurological deficits were evaluated prior to injury and at 1, 3 and 7 days post-injury. The expression of GAP-43, Akt, p-Akt, ERK1/2, and p-ERK1/2 proteins was measured by western blot analysis. ***Results:*** BMSC transplantation attenuated neurological deficits 3-7 days post-ICH. The expression of GAP-43 was increased 3 days following BMSC transplantation. However, this increase was inhibited by either PD98059 or LY294002 treatment. Treatment with both PD98059 and LY294002 was more effective than was treatment with an individual compound. ***Conclusion:*** BMSC transplantation could attenuate neurological deficits and activate axonal regeneration in this rat ICH model. The protective effects might be associated with increased GAP-43 expression by activating both the ERK1/2 and PI3K/Akt signaling pathways.

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Introduction

Intracerebral hemorrhage (ICH) is a life-threatening disease that accounts for 15–20% of all strokes [1]. Following treatment, most patients improve gradually during the first several months, but motor function recovery generally remains incomplete [2]. The motor deficits in a stroke result from an interruption of the motor fibers descending from the cortex to the spinal cord. Therefore, re-establishing connections between cortical neurons and their peripheral targets would provide a physical element for functional restoration.

The corticospinal tract (CST) is the key pathway controlling motor function, and the severity of CST damage is closely associated with the motor outcome in acute ICH [3]. It has been described that several factors regulated the outgrowth and guidance of CST axons [4, 5]. In particular, growth-associated protein-43 (GAP-43) is involved. This protein belongs to the calmodulin-binding protein family, is highly distributed in the presynaptic membrane [6] and is related to both long-term synaptic enhancement and long-term depression [7, 8]. Direct evidence has been provided in an *in vitro* study that GAP-43 expression increases gradually in the axon regeneration process [9]. Moreover, it also has been demonstrated that the most remarkable regenerative event was axonal sprouting, which occurred along with high GAP-43 expression in stroke models [10, 11]. Thus, we hypothesized that GAP-43 might be a prospective therapeutic target for treating ICH-induced motor deficits.

Stem cell transplantation is one potential therapy effective for various nerve injuries [12]. Bone marrow mesenchymal stem cells (BMSCs) could differentiate into various cell types because they are multi-potent and have a self-renewing capacity [13]. A previous study demonstrated that BMSC transplantation is effective in acute brain injury by increasing neurotrophic factors and promoting functional recovery [14]. It has been indicated that BMSCs have a therapeutic potential that is great for both ischemic and hemorrhagic strokes [15]. However, it is unclear whether BMSC transplantation promotes CST axonal regeneration and what mechanisms participate in the process.

To determine the roles of BMSC transplantation in ICH, a rat model of autologous blood injection was used. PD98059, a p-ERK1/2 inhibitor or/and LY294002, a PI3K inhibitor were selected for the mechanism study. We sought to test the hypothesis that BMSC transplantation regulates GAP-43 expression via the ERK1/2 and PI3K/Akt signaling pathways.

Materials and Methods

Animals and experimental groups

All experiments were in compliance with the guidelines of the National Institutes of Health for Care and Use of Laboratory Animals and were approved by the Hebei Medical University experimental ethics committee. Male rats (body weight 250–280 g) were supplied from the Experimental Animal Center of Hebei United University (Tangshan, Hebei, China) and were housed in the animal facility with a 12-hour day and night light cycle. They were allowed free access to food and water.

Experimental groups included vehicle, BMSCs, BMSCs + PD98059, BMSCs + LY294002, or BMSCs + PD98059 + LY294002 (n=15 per group). All animals were randomly assigned to the groups after blood injection.

Primary culture of BMSCs

The femurs were harvested from 21-day-old male Sprague-Dawley rats, and the medullary cavity was flushed with the Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Carlsbad, CA, USA). Bone marrow cells were collected, purified, and transferred into cell culture flasks (Corning Life Sciences, Tewksbury, MA, USA). Then, these cells were cultured in DMEM containing 10% fetal calf serum (Gibco-BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 37°C with 5% CO_2. Then, the medium was removed along with non-adherent cells. Fresh medium was added. This replacement was performed every 3 days. After the primary culture, the cells were sub-cultured at 1×10^4 cells/cm^2 and were used in the experiments after 3 passages.

ICH model

The rat ICH model was conducted as described in a previous study [16]. In brief, rats received a 100 mg/kg chloral hydrate i.p. injection and were mounted on a stereotaxic apparatus. A small hole was then drilled on the skull in a location that was 0.2 mm anterior to the bregma and 3.5 mm lateral to the midline. A needle was inserted into the right basal ganglia, 5.5 mm below the brain's surface under stereotactic guidance. One hundred microliters of autologous arterial blood was slowly infused with a micro-infusion pump at a speed of 5 μL/min. Upon completion of the infusion, the needle was left in place for 20 minutes. Bone wax was applied on the hole after removal of the needle. All procedures were conducted under aseptic conditions to avoid infection. In the mechanism experiment, injured rats received a lateral ventricular injection of 2 μg PD98059 (Beyotime Institute of Biotechnology, Jiangsu, China) and/or 1 μg LY294002 (Beyotime Institute of Biotechnology, Jiangsu, China) or PBS 10 min after ICH. The volume of each injection was 2 μL.

Transplantation of BMSCs

BMSCs at passage 4 were taken for transplantation and were dispersed into single cells. Then, 0.1 mL of cells (5 × 10) was intravenously given through a retro-orbital injection at 1 and 24 h after ICH, as previously reported [17]. The rats in the vehicle group were administered an equal volume of PBS.

Table 1. Neurological Severity Scores (NSS)

	Score
Raising rat by tail(normal=0; maximum=3)	(3)
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30s	1
Placing rat on floor (normal=0; maximum=3)	(3)
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests(normal=0; maximum=2)	(2)
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation)	1
Beam balance tests (normal=0; maximum=6)	(6)
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60s)	3
Attempts to balance on beam but falls off (>40s)	4
Attempts to balance on beam but falls off (>20s)	5
Falls off; no attempt to balance or hang on to beam (<20s)	6
Reflex absence and abnormal movements	(4)
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise)	1
Seizures, myoclonus, myodystony	1
Maximum points	(18)

Western blot analysis

Perihematoma brain tissue was lysed in Tissue Protein Lysis Solution (Life Technologies, Gent, Belgium) containing 5% Proteinase Inhibitor Cocktail (Sigma Aldrich, Diegem, Belgium), kept on ice for 30 min of incubation and then centrifuged at 15,000 g for 15 min. The bicinchoninic acid (BCA) protein assay (Jiancheng, Nanjing, China) was used to determine the protein concentration. Proteins in each sample were run on 10% SDS-PAGE and transferred to PVDF membranes that were blocked with 5% BSA for 1 h, followed by overnight incubation at 4°C in various antibodies, rabbit anti-rat GAP-43, Akt, p-Akt, ERK1/2, p-ERK1/2 and β-actin polyclonal antibody(1:1000 diluted, Santa Cruz, CA, USA). The membranes were washed the next day and were incubated in secondary antibodies (1:5000 diluted, Danvers, MA, USA). The bands were visualized with an enhanced chemiluminescent (ECL, Hercules, CA, USA) reagent and were quantified using the Image Quant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

Evaluation of neurological score

The Neurological Severity Score (NSS) was examined before surgery and 1, 3 and 7 days after ICH. The investigator who performed the test was blinded to the groups. NSS was graded using a scale of 1 to 18, in which the rat received a score of 1 point when he was not about to perform the test or lost a reflex (Table1).

Statistical analysis

All data are expressed as the mean ± SD and were analyzed using a one-way ANOVA followed by the Student-Newmann-Keuls post hoc test (SPSS 17.0 software). A statistically significant result is considered at $p < 0.05$.

Results

Neurological deficits in ICH were improved by BMSC transplantation

ICH induced significant neurological deficits compared with the pre-operated animals. Moreover, BMSC treatment decreased the NSS scores at 3 and 7 days post-transplantation ($p < 0.01$, vs. vehicle group). However, PD98059 or LY294002 treatment individually blocked the effect of BMSCs at 7 days following ICH ($p < 0.05$). Both the PD98059 and LY294002 treatments blocked the effect of BMSCs at 3 and 7 days after ICH ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 1).

Activation of ERK1/2 and PI3K/Akt pathway after BMSC transplantation

At 3 days following ICH, the expression of ERK1/2, p-ERK1/2, Akt and p-Akt were analyzed using western blot analysis. Compared to the vehicle group, the levels of p-ERK1/2 and p-Akt were significantly increased in BMSC transplantation groups ($p < 0.01$) (Fig. 2 and Fig. 3). As depicted in Fig. 2, PD98059 treatment dramatically inhibited p-ERK1/2 expression ($p < 0.01$, vs. BMSCs group). Furthermore, LY294002 treatment significantly attenuated p-Akt expression ($p < 0.01$, vs. BMSCs group) (Fig. 3).

Increase of GAP-43 expression after BMSC transplantation

To further investigate the underlying mechanism related to the effect of BMSC transplantation, the expression level of CST axonal regeneration related protein GAP-43 was analyzed in the tissue around the hematoma. As depicted in Fig. 4, transplantation of BMSCs dramatically increased GAP-43 protein expression ($p < 0.01$, vs. vehicle group). However, this increase inGAP-43 expression was attenuated in the PD98059 ($p < 0.05$), LY294002 ($p < 0.05$) and PD98059+LY294002 ($p < 0.01$) groups. Moreover, a combination treatment of

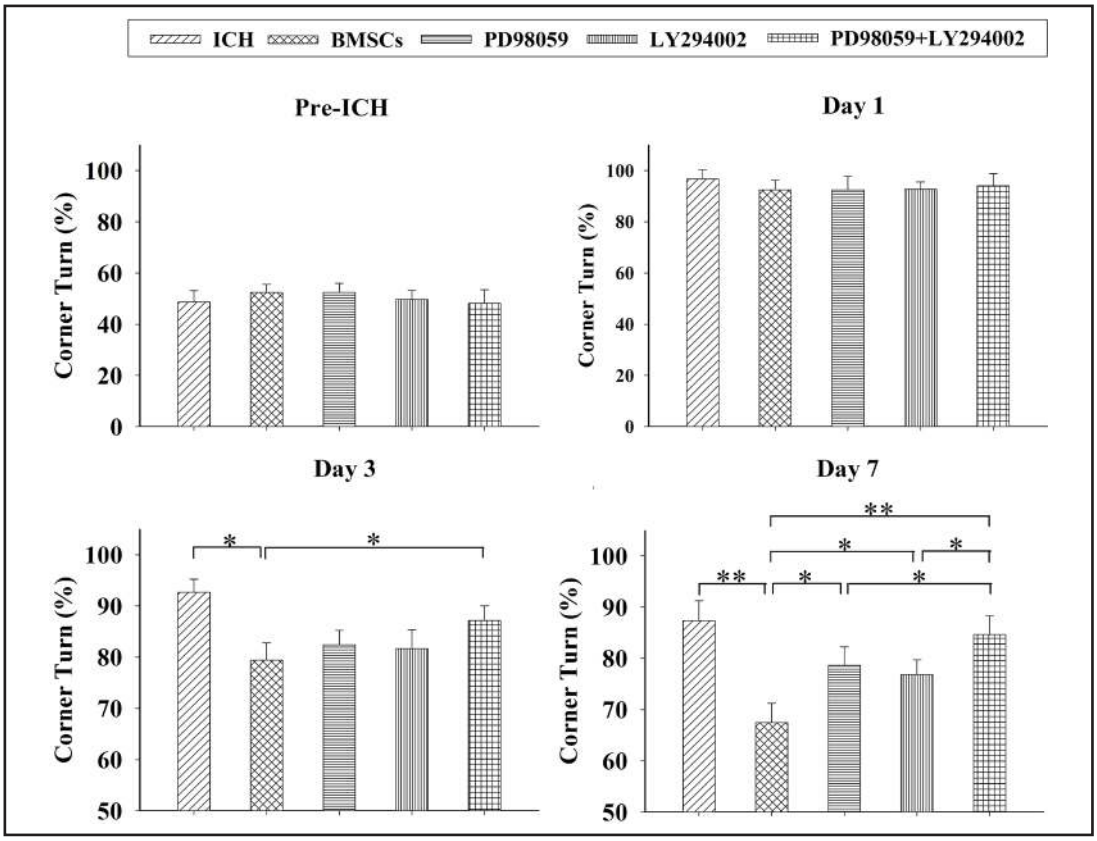

Fig. 1. NSS scores before and at 1, 3 and 7 days after ICH. Dates represent mean ± standard error (n=7, per group). *$p < 0.05$, **$p < 0.01$.

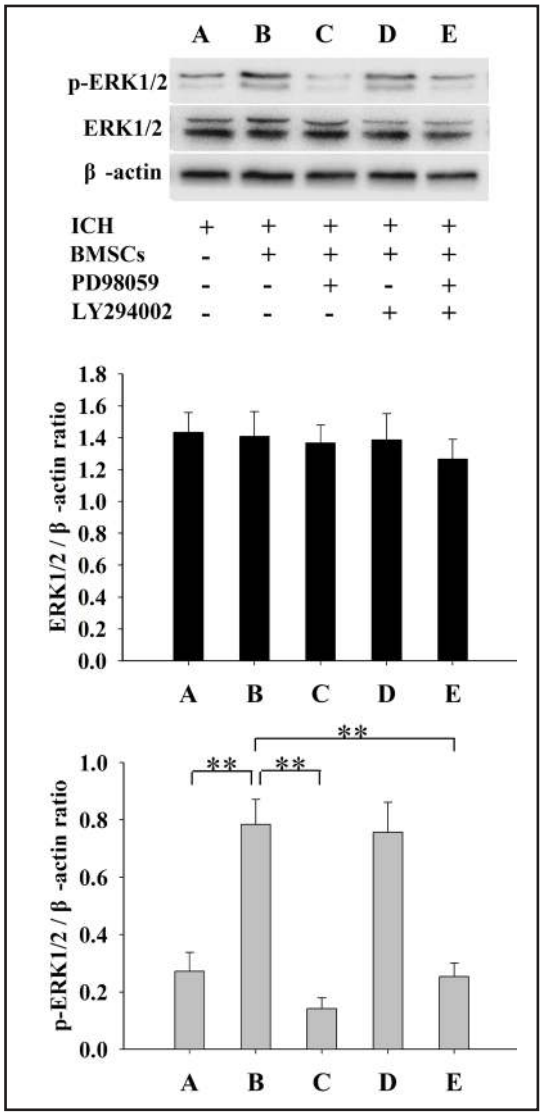

Fig. 2. The activation of ERK1/2 pathway after BMSC transplantation. Western blot analysis demonstrates levels of ERK1/2 and p-ERK1/2 in the perihematomal tissues of rats at 3 days ICH. The quantitative results of ERK1/2 and p-ERK1/2 were expressed as the ratio of the densitometries to β-actin bands, expressed as the mean ± standard error (n=8, per group). **p < 0.01.

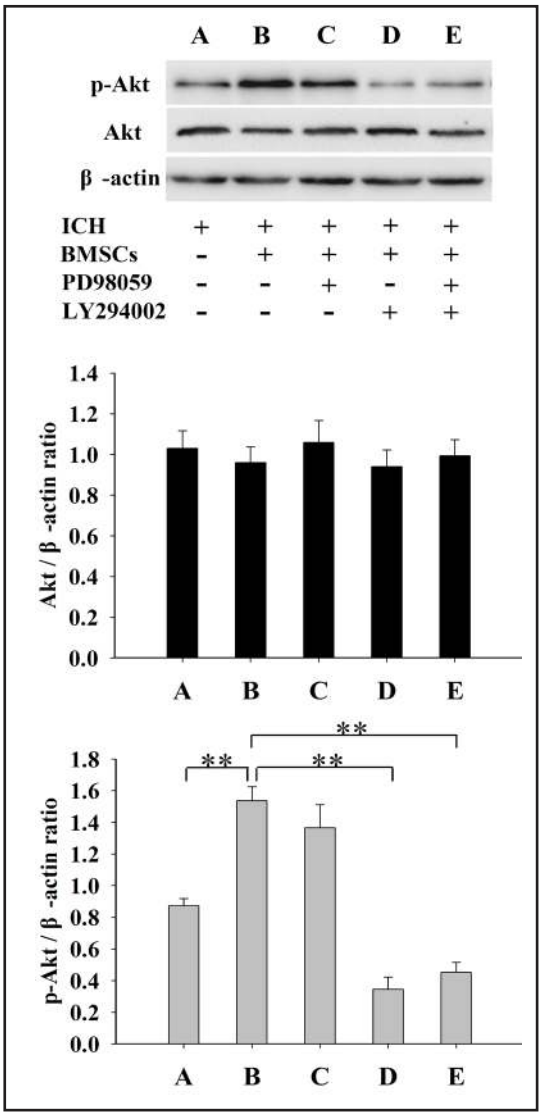

Fig. 3. The activation of PI3K/Akt pathway after BMSC transplantation. Western blot analysis demonstrates levels of Akt and p-Akt in the perihematomal tissues of rats at 3 days ICH. The quantitative results of Akt and p-Akt were expressed as the ratio of the densitometries to β-actin bands, expressed as the mean ± standard error (n=8, per group). **p < 0.01.

PD98059 and LY294002 was more effective than PD98059 or LY294002 treatment alone ($p < 0.05$) (Fig. 4).

Discussion

ICH is a complicated pathological status in the adult population [2]. Although many resources have been invested in clinical and basic research on ICH, there is still no effective drug that is currently available [18]. This study has demonstrated that BMSC transplantation

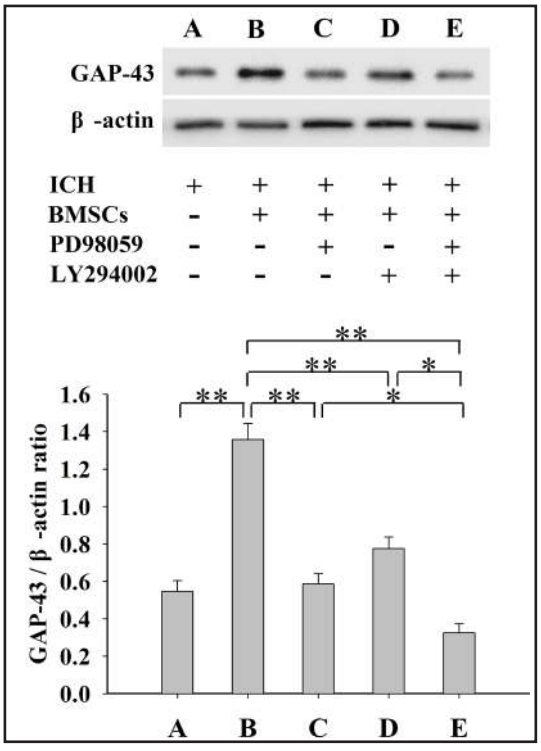

Fig. 4. Expression of GAP-43 increased after BMSC transplantation. Western blot analysis demonstrates levels of GAP-43 in the perihematomal tissues at 3 days post-ICH. The quantitative results of proteins were expressed as the ratio of the densitometries to β-actin bands, expressed as the mean ± standard error (n=8, per group). *p < 0.05, **p < 0.01.

attenuated neurological deficits 3-7 days post-ICH. At the molecular level, the expression of GAP-43 was found to increase in the area surrounding the hematoma at 3 days following BMSCs transplantation. Furthermore, we are the first to find that treatment with PD98059 or LY294002 blocked the protective effect of BMSCs. Treatment with PD98059 and LY294002 together resulted in further inhibition. These observations confirm our hypothesis that the neuroprotective effects of BMSCs in ICH might be the result of an increase of GAP-43 expression through the mechanism of both ERK1/2 and PI3K/Akt activation.

Although bacterial collagenase injection is another method that is widely used to induce ICH in rats, the model that we used has its own advantage in that the blood volume distributed into a location of the brain can be controlled. This application should be focused on studies of blood toxicity because of the absence of some key pathological aspects of ICH, such as arterial rupture and hematoma expansion [19]. ICH is commonly induced in the striatum area. Nevertheless, hematoma is seen more often in the basal ganglia than in the striatum in patients. Therefore, we have chosen to inject blood into the basal ganglia in this study.

Stem cells could modify the tissue microenvironment by secreted solvable factors, including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and Insulin-like growth factor-1 (IGF-1) [20, 21]. Several studies have demonstrated that these factors could promote axonal regeneration [21, 22]. Andres and colleagues identified that the neutralization of VEGF inhibited axonal sprouting that was enhanced by stem cell transplantation [23]. Gupta and colleagues showed that GAP-43 is a key mediator contributing to the neurotrophic effects of BDNF on neuronal survival and neurite outgrowth [24]. Moreover, Liu and colleagues reported that IGF-1 might have a neuroprotective effect by regulating GAP-43 expression. The action was completed through both ERK1/2 and PI3K/Akt activation [25]. Our findings are in conformity with Liu's report and suggest that CST axonal regeneration is mediated by soluble factors secreted from BMSCs in the study. However, it may not be the only mechanism underlying the therapeutic effects of BMSCs on GAP-43. The actions are currently recognized to be more complicated and are not well understood. Differentiation of BMSCs was involved in axonal regeneration as well [26]. In addition, BMSCs were found to exert anti-inflammatory effects at the injury site as they

modulated the activity of dendritic cells [27]. The resulting decreased inflammatory effects might influence axonal regeneration indirectly under these circumstances. Therefore, this topic requires further investigation during the later phase following ICH.

Conclusion

In summary, the results from this study indicated that BMSC transplantation attenuated neurological deficits and activated axonal regeneration in ICH. These neuroprotective effects might be derived from an increase of GAP-43 expression through ERK1/2 and PI3K/Akt activation in which the signaling pathways in these stem cells were modulated.

Disclosure Statement

None.

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