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Effects of recombinant adenovirus-mediated hypoxia-inducible factor-1alpha gene on proliferation and differentiation of endogenous neural stem cells in rats following intracerebral hemorrhage

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ABSTRACT

Objective: To investigate the effects of adenovirus (Ad)-mediated hypoxia-inducible factor-1alpha (HIF-1 α) gene on proliferation and differentiation of endogenous neural stem cells (NSCs) in rats following intracerebral hemorrhage (ICH) and the underlying mechanisms. **Methods:** A total of 120 specific pathogen-free, adult, male Sprague-Dawley rats were included in this study. After establishment of ICH models in rats, PBS, Ad, or Ad-HIF-1 α was administered via the ischemic ventricle. On the 1st, 7th, 14th, 21st and 28th d after ICH, rat neurological deficits were scored, doublecortin (DCX) expression in the subventricular zone cells was detected by immunohistochemical staining, and 5-bromo-2′-deoxyuridine (BrdU)-, BrdU/DCX-, and BrdU/glial fibrillary acidic protein-positive cells in the subventricular zone were counted using immumofluorescence method among PBS, Ad, and Ad-HIF-1 α groups. **Results:** On the 7th, 14th, 21st and 28th d after ICH, neurological deficit scores in the Ad-HIF-1 α group were significantly lower than in the PBS and Ad groups (P<0.05). In the Ad-HIF-1 α group, DCX expression was significantly increased on the 7th d, peaked on the 14th d, and then gradually decreased. In the Ad-HIF-1 α group, BrdU-positive cells were significantly increased over time course, and significant difference in BrdU-positive cell counts was observed when compared with the PBS and Ad groups at each time point (P<0.01 or 0.05). On the 7th, 14th, 21st and 28th d after ICH, the number of DCX-, BrdU-, BrdU/DCX-, and BrdU/DCX-positive cells in the Ad-HIF-1 α group was significantly greater than in the PBS and Ad groups (P<0.05). **Conclusions:** HIF-1 α gene can promote the proliferation, migration and differentiation of endogenous neural stem cells after ICH, thereby contributing to neurofunctional recovery after ICH.

1. Introduction

Ischemic tolerance is the most powerful endogenous protective mechanism against ischemia injury. Hypoxia-inducible factor-1alpha (HIF-1 α) is a transcription factor closely related to ischemic tolerance. There is evidence that HIF-1 α exhibits strong protective effects on hypoxic-ischemic brain damage by promoting anaerobic metabolism of brain cells, vasoformation, oxygen transport of erythrocytes, improving energy metabolism disorder, haemodynamic recovery, and reducing cell apoptosis after cerebral ischemia[1]. The mechanism by which HIF-1 α protects endogenous neural stem cells (NSCs) from cerebral hemorrhage remains poorly understood. In this study, we injected recombinant adenovirus (Ad) carrying HIF-1 α

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gene (Ad-HIF-1 α) into the hemorrhagic ventricle of rat models of intracerebral hemorrhage (ICH) established by autologous blood injection, labeled cells in S phase with 5-bromo-2′-deoxyuridine (BrdU), scored neurological deficits and detected DCX and BrdU expression in the hemorrhagic tissue by immunohistochemical staining. We studied the effects of HIF-1 α gene on proliferation, migration and differentiation of endogenous NSCs and the underlying mechanism, correlated HIF-1 α with cerebral ischemia, and investigated the therapeutic effects of recombinant adenovirus-mediated HIF-1 α on ICH in rats, providing a new method for the treatment of ICH.

2. Materials and methods

2.1. Animals

A total of 120 specific pathogen-free, adult, male Sprague-Dawley rats, weighing 270-320 g, provided by Laboratory Animal Center, Third Military Medical University of Chinese PLA, were included in this study. All experimental procedures were performed in strict accordance with the Regulations for the Administration of Affairs concerning Experimental Animals issued by the State Council of China in 1988. Three groups, Ad-HIF-1 α, Ad and PBS, were designated (n = 40). Immediately after establishing ICH models, rats in the Ad-HIF-1 α, Ad and PBS groups received 10 μL of Ad-HIF-1 α (1×10^8 pfu), Ad (1×10^8 pfu) or sterile PBS respectively via the ischemic ventricle.

2.2. Main reagents and instruments

Ad empty vector and Ad-HIF-1 α were gifted by Dr. Tao Tao from Department of Neurology, Second Affiliated Hospital of Chongqing Medical University, China. Nestin BD was purchased from BD Biosciences Pharmingen (San Diego, CA, USA), glial fibrillary acidic protein (GFAP) from Thermo Fisher Scientific Inc., (Rockford, IL, USA), NeuN from Chemicon (Billerica, MA, USA), doublecortin (DCX) from Cell Signaling Technology Inc., (Danvers, MA, USA), and 5-bromo-2′-deoxyuridine (BrdU) from Sigma (St. Louis, MO, USA). Rat stereotaxic instrument was provided by Xi′an Optical Medical Instrument Co., Ltd., (Xi′an, China) and microinjector by Shanghai Anting Microsyringe Factory (Shanghai, China).

2.3. Establishment of ICH models in rats

After anesthesia, rats were placed in a stereotaxic instrument in the prone position. According to a previously described method, a medial skin incision was made over the skull to expose the bregma. A pore with a diameter of approximately 1 mm was drilled at 0.2 mm anterior to the bregma and 3.5 mm right lateral to the midline. Rat tail was cut off at 5 mm away from the tail end and 75 μL blood was taken through the use of microinjector to establish ICH models with similar sized hematomas. Then, the microinjector was fixed in the stereotaxic instrument and inserted into the pore at the depth of 5.5 mm and maintained in place for 10 min.

2.4. Stereotaxic injection into the ventricle

Rats were administered 10 μL of PBS, Ad or Ad-HIF-1 α using the microinjector into the site 1.5 mm posterior to bregma, 2 mm right lateral to the midline, and 3.5 mm below the dura mater within 10 min. The microinjector was left in place for 10 min and then slowly withdrawn. The pore was sealed with bone wax and scalp was sutured layer by layer. The local skin was disinfected with iodine tincture for preventing infection. Rat body temperature, heart rate and respiratory frequency were monitored continuously throughout the experiment.

2.5. BrdU labeling

BrdU solution (5×10^3 g/L) prepared by saline was intraperitoneally administered at 50 mg/kg, once every 12 h, for a total of 2 successive days. Rats were sacrificed 24 h after the last BrdU (5×10^3 g/L) administration for detecting cell proliferation changes of normal cells and injured cells over time course. BrdU solution (5×10^3 g/L) prepared by saline was intraperitoneally administered at 50 mg/kg 12 h after ICH, once per day, for a total of 14 d. Rats were sacrificed 14 d after the last BrdU solution (5×10^3 g/L) administration (*ie.*, day 28 after ICH) for detecting the differentiation of new generally cells.

2.6. Assessment on improvement in neurological functions

On the 1st, 7th, 14th, 21st and 28th d after surgery, rat neurological deficits were scored using Neurological Severity Score (NSS). Rats that could not perform above tasks or lacked of corresponding reflex were scored 1. Scores 1-6 indicate mild neurological deficits, 7-12 moderate neurological deficits, and 13-18 severe deficits.

2.7. Preparation of tissue sections

At postoperative 4 h, 1, 3, 7 d, rat thoracic cavity was opened to expose the heart under anesthesia by 10% chloral hydrate (0.35 mL/100 g). The needle was inserted into the aorta from the cardiac apex and via the left ventricle and then fixed. An aliquot of 200 mL normal saline was rapidly perfused and simultaneously a cut was made in the right atrium. After blood was washed away, 300 mL 4% paraformaldehyde was perfused at 4 ℃. Following craniotomy, the brain was harvested and fixed in the fixing solution for 48 h at 4 ℃. Then the brain tissue was

transferred to 30% sucrose until it sank to the bottom. After one PBS wash and OCT embedding, a brain tissue block spanning across 3 mm anterior to 5 mm posterior from anterior border of the optic chiasma was removed through the use of freezing microtome. The brain tissue block, containing cerebral cortex and dorsal caudate putamen, was sliced into 16 μm thick coronal sections.

2.8. DCX and BrdU immunohistochemical staining

Tissue sections were washed with 0.01 M PBS three times for 5 min each, incubated with 3% H_2O_2 for 15-30 min to inactivate endogenous peroxidase, blocked with normal goat serum for 30 min and then incubated overnight after addition of rabbit anti-DCX (1: 200) and anti-BrdU (1: 500) antibodies. After three washes with 0.01 M PBS for 5 min each, tissue sections were incubated with biotinylated goat anti-rabbit IgG (1:100) or goat anti-IgG (1:100) antibodies at 37 ℃ for 3 h. After three washes with 0.01 M PBS for 5 min each, tissue sections were incubated with SABC (1:100) at 37 ℃ for 2 h. Then, tissue sections were developed with 3,3′-diaminobenzidine, dehydrated in ethanol gradients, cleared , mounted with neutral gel and finally observed under optical microscope. For negative control, PBS served as primary antibody, and the remaining procedures were the same as above.

2.9. Double immunofluorescent labeling

Tissue sections were washed with 0.01 M PBS three times for 5 min each, treated with 0.1% Triton for 30 min, blocked with normal goat serum for 30 min, incubated with primary antibody rat anti-BrdU antibody (1: 500) at 4 ℃ overnight, with fluorescent secondary antibody goat ant-rabbit TRITC (1:100) at 37 ℃ for 1 h, with primary antibodies rabbit anti-DCX (1:200), rabbit ant-GFAP (1:200) or rabbit anti-CD31 (1:200) antibodies at 37 ℃ for 1 h in the dark, and with fluorescent secondary antibody goat anti-rat FITC (1:100) antibody. Three PBS washes for 5 min each were used for all intermittent steps. Then tissue sections were left at room temperature in the dark for 2 h and air dried. Finally, they were observed under the fluorescent microscope. For the negative control, PBS served as the primary antibody, and the remaining procedures were the same as above.

2.10. Immunohistochemical observation results and statistical analysis

Five rats were selected from each group for immunohistochemical observation. Ten sections per rat were randomly selected. Under 200-fold magnification, five fields of view in each section were randomly selected for cell counts. All data were expressed as mean±SD and statistically processed with SPSS 11.5 software. Analysis of variance was used for comparison between groups. *t*-test was used for pairwise comparison at the same time point. A level of α =0.05 was considered statistically significant.

3. Results

3.1. Neurological deficit assessment by NSS

There was no significant difference in neurological deficit scores at 1 d after ICH between Ad-HIF-1 α and Ad groups ($P > 0.05$), but significant differences existed between Ad-HIF-1 α and PBS groups or between Ad and PBS groups ($P < 0.01$). At 7-28 d after ICH, neurological deficit scores in the Ad-HIF-1 α group were significantly lower than in the Ad and PBS groups (both $P < 0.01$), while there was no significant difference in neurological deficit scores between Ad and PBS groups (all $P > 0.05$) (Figure 1).

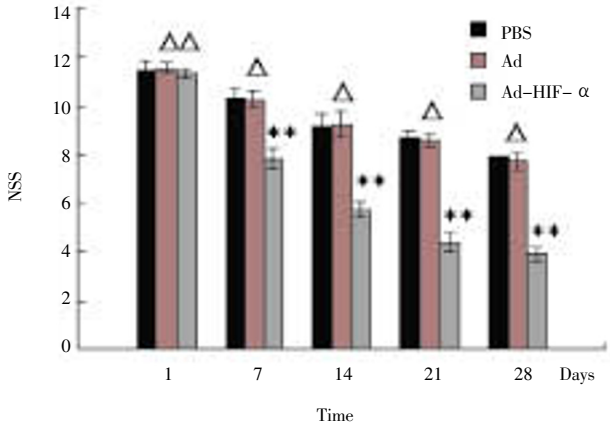

Figure 1. Comparison of NSS among groups.
*P<0.01 for PBS group *vs.* Ad group; △$P > 0.05$ for Ad group *vs.* PBS groups.

3.2. DCX immunohistochemical staining results

DCX-positive cells were primarily located in the ipsilateral subventricular zone and the tissue surrounding the corpus callosum. They were slightly increased on the 3rd d after ICH, significantly increased on the 7th d, peaked on the 14th d, and then gradually decreased. On the 7th, 14th and 28th d after ICH, the number of DCX-positive cells in the Ad-HIF-1 α group was significantly greater than in the Ad group ($P < 0.05$) (Figures 2-5).

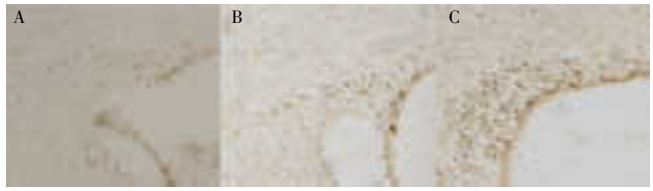

Figure 2. Immunohistochemical detection of DCX-positive cells in the subventricular zone after intracerebral hemorrhage.
From left to right (A-B-C): PBS, Ad and Ad-HIF-1 α groups, respectively. Bar=50 μm.

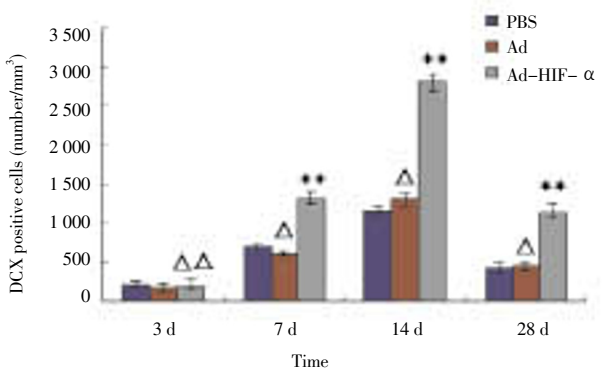

Figure 3. Comparison of DCX-positive cells in the subventricular zone at different time points after intracerebral hemorrhage.
(*P<0.01 for Ad-HIF-1 α group *vs.* PBS group; #P<0.01 for Ad-HIF-1 α group *vs.* Ad group; △P >0.05 for Ad group *vs.* PBS group.

Figure 4. Immunohistochemical detection of DCX in the corpus callosum after ICH.
From left to right (A-B-C): PBS, Ad, Ad-HIF-1 α groups respectively. Bar=50 μm.

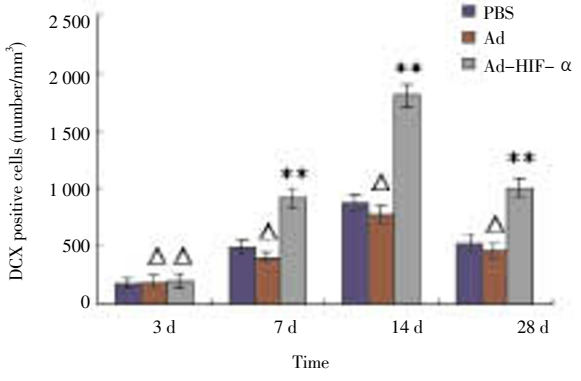

Figure 5. Comparison of DCX-positive cells in the corpus callosum of rats from each group at different time points after intracerebral hemorrhage.
*P<0.01, #P<0.05, △P>0.05 for PBS group *vs.* Ad group; *P<0.01, #P<0.05, △P>0.05 for Ad group *vs.* PBS group.

3.3. BrdU immunohistochemical staining results

BrdU-positive cells could be seen in the subventricular zone of rats in the PBS, Ad and Ad-HIF-1 α groups. In each group, BrdU-positive cells increased on the 3rd d after ICH, peaked on the 7th d and gradually decreased after 14 d. In the Ad-HIF-1 α group, BrdU-positive cells were significantly increased over time course, and significant difference in BrdU-positive cell counts was observed when compared with the PBS and Ad groups at each time point (P < 0.01 or 0.05) (Figure 6, 7).

Figure 6. Immunohistochemical detection of BrdU in the subventricular zone of rats from each group after intracerebral hemorrhage.
From left to right (A-B-C): PBS, Ad and Ad-HIF-1 α groups, respectively. Bar=50 μm.

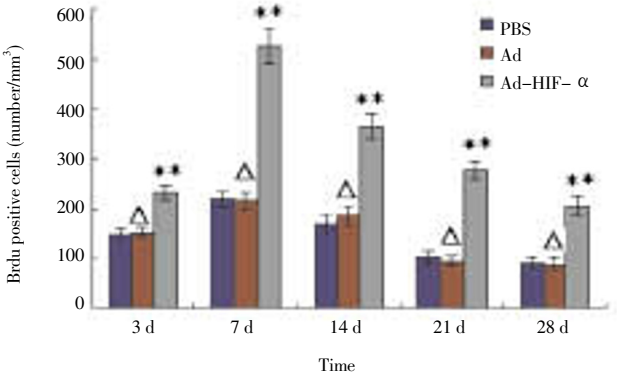

Figure 7. Comparison of BrdU-positive cells in the subventricular zone of rats from each group at different time points after intracerebral hemorrhage.
**P<0.05 for PBS group *vs.* Ad group; △P>0.05 for Ad group *vs.* PBS group.

3.4. BrdU/DCX and BrdU/GFAP immunofluorescent double staining results

The differentiation of newly generated cells was detected by BrdU/DCX and BrdU/GFAP immunofluorescent double staining method. Results showed that BrdU/DCX- and BrdU/GFAP-positive cells were observed in the subventricular zone of rats in each group on the 28th d after ICH, and the number of BrdU/DCX- and BrdU/GFAP-positive cells in the Ad-HIF-1 α group was significantly greater than that in the Ad group (P < 0.05) (Figure 8-11).

Figure 8. DCX/BrdU double immunofluorescent staining of the peri-hematoma tissue under the confocal laser scanning electron microscope.
(A) DCX-positive cells; (B) BrdU-positive cells; (C) merge of (A) and (B). Nuclei were stained with DAPI. Bar=50 μm.

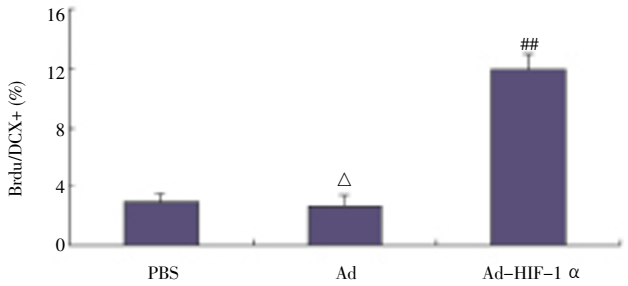

Figure 9. Comparison of BrdU/DCX-positive cells in the peri-hematoma tissue of rats in each group at day 28 after intracerebral hemorrhage.
#P<0.05 for PBS group *vs.* Ad group; △P>0.05 for Ad group *vs.* PBS group.

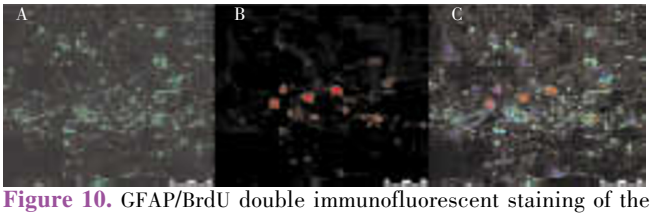

Figure 10. GFAP/BrdU double immunofluorescent staining of the peri-hematoma tissue under the confocal laser scanning electron microscope.
(A) GFAP-positive cells; (B) BrdU-positive cells; (C) merge of (A) and (B). Nuclei were stained with DAPI. Bar=50 μm.

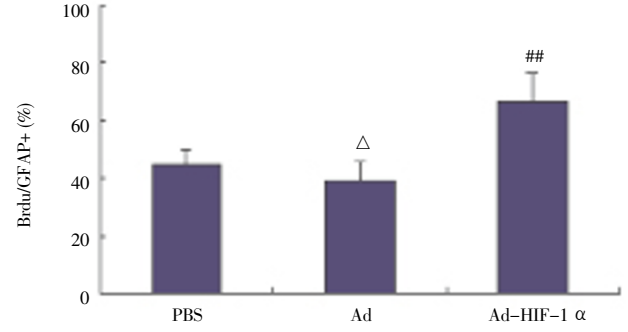

Figure 11. Comparison of BrdU/GFAP-positive cells in the peri-hematoma tissue of rats in each group at day 28 after intracerebral hemorrhage.
#P<0.05 for PBS group *vs.* Ad group; △P>0.05 for Ad group *vs.* PBS group.

4. Discussion

Endogenous HIF-1 α has no ability to protect the brain against cerebral hemorrhage because of its not enough expression and short action time. Recombinant adenovirus-transfected HIF-1 α gene can maintain high level of HIF-1 α in the peri-hematoma brain tissue, infect differentiated and undifferentiated cells, and thereby exhibit therapeutic effects on ICH.

Studies have demonstrated that neural precursor cells exist in the subventricular zone, hippocampal dentate gyrus, and cerebral cortex[2,3]. All kinds of brain injuries, including cerebral infarction, can induce neurogenesis[4,5]. Neurogenesis following experimental cerebral infarction can facilitate recovery from brain injuries[6]. Neurogenesis can be induced in rat models of subarachnoid hemorrhage or collagenase-induced ICH[7,8]. Our results also demonstrated that neurogenesis could be also induced in rat models established by autologous blood injection and lasted over 4 weeks. This suggests that neurogenesis exists long time after ICH and HIF-1 α exerts an important role in ICH-induced neurogenesis.

HIF-1 α plays an important role in the proliferation of embryonic stem cells and the development of fetal rats; HIF-1 α gene knockout can cause death of a large number of brain cells and neural tube defects[9]. HIF-1 α gene knockout can cause developmental brain defects, reduce neural cells in quantity and induce spatial memory disorder in mice[10]. HIF-1 α gene deletion in the mesencephalic neural stem cells reduced cell survival and differentiation[11]. Under hypoxic condition, HIF-1 α signal transduction pathway can promote the proliferation and differentiation of NSCs[12]. This suggests that HIF-1 α is the essential physiological condition for the development of nervous system.

Erythropoietin (EPO), a target gene for HIF-1 α, exhibits protective effect on nerve cells, effectively alleviates pathological cerebral damage, and also induces neurogenesis after brain injury. EPO greatly reduces the infarct area of rats with cerebral infarction, maintains learning and memory abilities and significantly improves prognosis[13]. EPO can effectively reduce neuronal apoptosis, enlarge the soma of surviving neurons, increase neuronal neurites and strength their functions after cerebral ischemia[14].

Many factors, including vascular endothelial growth factor, mediate post-stroke neurogenesis. VEGF is a specific potent mitogen for endothelial cells and a potent stimulator of vascular regeneration. Recent studies have demonstrated that VEGF plays an important role in neurogenesis. Wang *et al*[18] reported that VEGF can promote axon outgrowth in cultured cortical neurons. There is evidence that VEGF reduces infarct volume, promotes the proliferation and migration of neural progenitor cells in the subventricular zone, and thereby contributes to neurofunctional recovery[19]. Neurovascular niche formation plays an important role in neurogenesis after brain injury.

After ICH, endogenous NSCs in the subventricular zone and hippocampal dentate gyrus are activated, and the newly generated cells migrate toward peri-hematoma brain tissue and differentiate into diverse neural cells[21]. Results from this study showed that in the Ad-HIF-1 α group, DCX-positive cells in the subventricular zone significantly increased in quantity at 7 d after ICH, peaked at 14 d and then decreased gradually; a large number of DCX-positive cells migrated toward peri-hematoma brain tissue during this time period. In the Ad-HIF-1 α group, the number of BrdU-positive cells in the subventricular zone increased at 3 d after ICH, peaked at 7 d and gradually decreased thereafter. The number of BrdU-positive cells in the Ad-HIF-1 α group was significantly higher than in the PBS and Ad groups. This occurs possibly because HIF-1 α can activate various target genes, promote the establishment of collateral circulation and improve cerebral microenvironment after ICH.

BrdU and DCX are two common markers used in the

study of neurogenesis. BrdU can be used to label cells in the S-phase of the cell cycle. A recent study has shown that BrdU can also label apoptotic cells. Therefore, DCX, a marker of new neurons, was used in this study to co-label new neurons. In the PBS group, BrdU/DCX-positive cells were not detected. In the Ad-HIF-1 α group, BrdU/DCX-positive cells were detected in the subventricular zone and peri-hematoma brain tissue, accounting for appropriately 12% of total number of proliferated cells, and about 67% proliferated cells differentiate into glial cells. Taken together, endogenous neural stem cells proliferated, migrated and differentiated in rat models of ICH established by autologous blood injection, and HIF-1 α gene plays an important role in neurogenesis after ICH.

This study established rat models of ICH established by autologous blood injection and scored neurological deficits of rats after Ad-HIF-1 α administration into the hemorrhagic ventricle to investigate the effects of recombinant adenovirus-mediated HIF-1 α gene on the proliferation, migration and differentiation of endogenous NSCs in the brain tissue and the possible mechanisms. Results showed that Ad-HIF-1 α gene therapy improves the microenvironment of peri-hematoma tissue by up-regulating HIF-1 α , VEGF and EPO expression levels, promotes the proliferation, migration and differentiation of endogenous NSCs, and thereby contribute to neurofunctional recovery after ICH.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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