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Granulocyte colony-stimulating factor improves neurological function and angiogenesis in intracerebral hemorrhage rats

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Abstract. – OBJECTIVE: Granulocyte colony-stimulating factor (G-CSF) plays a role in regulating phosphatidylinositol-3-kinase/serine/threonine kinase (PI3K/AKT) pathway, affecting cell proliferation and apoptosis, and inducing vascular endothelial growth factor (VEGF) expression. This study investigated the mechanism of G-CSF on angiogenesis and neural protection after intracerebral hemorrhage (ICH).

MATERIALS AND METHODS: The rats were divided into four groups, including sham, ICH, ICH+G-CSF, and ICH+G-CSF+LY294002 (PI3K/AKT signaling pathway specific inhibitor). Cerebral neurological dysfunction was tested by Garcia scoring. Cell apoptosis was detected by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. Angiogenesis marker CD34 expression, PI3K/AKT signaling pathway, B-cell lymphoma-2 (Bcl-2), and VEGF expressions were compared by IHC. Rat cerebral nerve RN-c cells were divided into four groups, including control, oxygen-glucose deprivation (OGD), OGD+G-CSF, and OGD+G-CSF+LY294002.

RESULTS: Neurological dysfunction was more evident; CD34+ cell number, VEGF expression, and cell apoptosis significantly increased; phosphorylated AKT (p-AKT) and Bcl-2 levels markedly reduced in ICH group compared with sham group. G-CSF apparently up-regulated p-AKT and Bcl-2 expressions, attenuated cell apoptosis, and elevated CD34+ cell number. LY294002 significantly decreased p-AKT, Bcl-2, and VEGF expressions, and alleviated the cell apoptosis protective and angiogenesis effect induced by G-CSF. OGD treatment induced RN-c cell apoptosis, down-regulated p-AKT and Bcl-2 expressions, and enhanced the tube capacity of vascular endothelial cells (VEC). G-CSF markedly elevated p-AKT and Bcl-2 contents in RN-c cells, declined cell apoptosis, increased p-AKT and VEGF levels in VEC, and enhanced tube capacity.

CONCLUSIONS: G-CSF enhanced PI3K/AKT signaling pathway activity, promoted Bcl-2 and VEGF expression, reduced nerve cell apoptosis, and enhanced tube capacity of VECs, which may be the mechanism of G-CSF in improving neurological function and angiogenesis after ICH.

Key Words:
Intracerebral hemorrhage, PI3K/AKT, Apoptosis, Angiogenesis, G-CSF.

Introduction

Intracerebral hemorrhage (ICH) refers to the primary non-traumatic parenchymal hemorrhage, with a characteristic of sudden onset, critical condition, high morbidity, and mortality rates that brings serious influence to health and quality of life[1-3].

Apoptosis (AP) is one of the important mechanisms of neurologic deficits after a cerebral hemorrhage. Alleviation of cell apoptosis has become an approach for neural function protection after ICH[4,5]. Granulocyte colony-stimulating factor (G-CSF) is a growth factor that plays an important role in promoting hematopoietic growth and differentiation[6]. Also, to regulate blood cells growth, G-CSF also has a neural protective function. Immune regulation and cell apoptosis inhibition are the crucial mechanisms of G-CSF to protect nerve function after ICH or cerebral ischemia[7-10]. As a classic pathway in antagonizing apoptosis and promoting cell proliferation, phosphatidylinositol 3 kinase/serine/threonine kinase (PI3K/AKT) signaling pathway exists in various tissues and cells[11,12]. It was showed that PI3K/AKT signaling pathway plays a key role in up-regulating B-cell lymphoma-2 (Bcl-2) expression, antagonizing cell apoptosis, and facilitating cell

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survival. Activation of PI3K/AKT signaling pathway exhibits an effect to protect nerve cell and promoting neurological function recovery, thus, it is a potential target for drug treatment[13,14].

Angiogenesis is an important compensatory mechanism to protect brain nerve function after ICH. Vascular endothelial growth factor (VEGF) plays a crucial role in promoting vascular endothelial cell proliferation, differentiation, and angiogenesis. It was reported that G-CSF can induce VEGF expression and secretion through regulating PI3K/AKT signaling pathway[15]. Though G-CSF plays a role in protecting brain nerve function defect and promoting angiogenesis, it is still controversy whether through the PI3K/AKT signaling pathway. This study investigated the mechanism of G-CSF on angiogenesis and neural protection after ICH.

Materials and Methods

Main Reagents and Instruments

Rat recombinant G-CSF was purchased from Peprotech Co. Ltd. (Rocky Hill, NJ, USA). Rat cerebral VEC was purchased from Jining Shiye (Shanghai, China). Rat cerebral neuron RN-c was bought from Yubo Biological Technology (Shanghai, China). Dulbecco's modified eagle medium (DMEM) medium, fetal bovine serum (FBS), neurobasal medium, B27, and GlutaMAX were got from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). RNA extraction reagent TRIzol was obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). Real-time PCR kit QuantiTect SYBR Green RT-PCR kit was provided by Qiagen (Hilden, Germany). Rabbit anti-rat AKT polyclonal antibody (Catalogue No. ab8805), rabbit anti-rat phosphorylated AKT polyclonal antibody (p-AKT, Catalogue No. ab81283), rabbit anti-rat Bcl-2 polyclonal antibody (Catalogue No. ab59348), and rabbit anti-rat β-actin polyclonal antibody (Catalogue No. ab8227) were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-rat VEGF polyclonal antibody (Catalogue No. 2463) was got from Cell Signaling Technology Inc. (Beverly, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibody was purchased from Boster (Wuhan, China). RIPA lysis buffer and Annexin V/PI apoptosis kit were got from Beyotime Biotech. (Shanghai, China). Matrigel was bought from BD Biosciences (Franklin Lakes, NJ, USA). The PI3K/AKT signaling pathway specific inhibitor LY294002 was obtained from MedchemExpress (Monmouth Junction, NJ, USA). Fluorescence microscope (DM3000) was got from Leica (Frankfurt, Germany). Cell incubator (DHP-9012) was provided by Daoxi Industrial Ltd. Co. (Shanghai, China). Flow cytometry (CytoFLEX) was purchased from Beckman Coulter Inc. (Brea, CA, USA). Real-time PCR amplifier (CFX96) was got from Bio-Rad Laboratories (Hercules, CA, USA).

ICH Model Establishment and Grouping

Sprague-Dawley (SD) rats (weighted 200-250 g, 8-week old, male, purchased from the Experimental Animal Center of Guangdong Medicine) were fasted for 12 h before the operation. After anesthetized by 1% pentobarbital sodium peritoneal injection at 40 mg/kg, the rat was fixed on stereotaxic apparatus in prone position. An incision was made in the brain to expose the bregma. The micro-injector was placed at 1 mm anterior to the bregma and 3 mm from the right midcourt line and inserted for 5 mm. A total of 50 μl autologous arterial blood was obtained from the caudal vessel. 10 μl blood was injected through the micro-injector and the left 40 μl blood was further injected after 2 min pause. The bone was blocked by bone wax and the skin incision was sutured. Penicillin was locally smeared to prevent infection. The rat in the sham group received 50 μl normal saline instead of autologous blood. This investigation was approved by the Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang, Heilongjiang.

SD rats were divided into four groups with 10 in each group, including sham, ICH, ICH+G-CSF, and ICH+G-CSF+LY294002 groups. The rats in the ICH group received an equal amount of normal saline peritoneal injection at 1 h after modeling every 24 h for three times. The rats in the ICH+G-CSF group received 40 μg/kg recombinant G-CSF peritoneal injection at 1 h after modeling every 24 h for three times. The rats in the ICH+G-CSF+LY294002 group received 40 μg/kg recombinant G-CSF and 30 μg/kg LY294002 peritoneal injection at 1 h after modeling every 24 h for three times.

Moisture Content in the Brain Tissue

The rats were anesthetized by 1% pentobarbital sodium peritoneal injection at 72 h after modeling. The brain tissue was extracted and measured for wet weight after removing the pia mater and bloodstain. Then, the tissue was baked at 100°C for 24 h. After that it was measured for dry weight.

The Moisture content was calculated according to the Billot formula. Brain moisture content (%) = (wet weight – dry weight)/wet weight ×100%.

Neurological Dysfunction Scoring

The Garcia score was evaluated at 24 h, 48 h, and 72 h after the operation. Garcia score assessed the neurological dysfunction degree from six aspects, including autonomic exercise, symmetry, forelimb extension function, screen experiment, bilateral tactile sensation, and bilateral beard reflex. The minimal and maximal score of each aspect was 0 and 3. The total score was 3-18. Lower total score referred to more severe neurological functional damage.

Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick end Labeling (TUNEL) Assay

The rats were anesthetized by 1% pentobarbital sodium peritoneal injection at 72 h after modeling. The brain tissue was extracted and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) to prepare paraffin section. The tissue section was dewaxed in xylene for 5-10 min and dehydrated by ethanol. Then, the tissue was added with 20 μg/ml proteinase K without DNase and incubated at 37°C for 20 min. Next, the tissue was washed by phosphate buffered saline (PBS) and incubated in TUNEL detection liquid prepared by 5 μl TdT enzyme and 45 μl fluorescence marker at 37°C avoid of light for 60 min. At last, the slice was blocked by anti-quenched peptide after washing by PBS and observed under the microscope. The mean apoptotic cell number under each visual field was calculated.

IHC Detection of Angiogenesis

The rats were anesthetized by 1% pentobarbital sodium peritoneal injection at 72 h after modeling. The aorta was perfused by 4% paraformaldehyde and the rat was killed. IHC method was applied to test $CD34^+$ positive cells. $CD34^+$ positive cells mainly locate in the endothelial cell of neovascularization. Endothelial cells or clusters stained as tan or brown were considered as $CD34^+$ positive, representing one neovascularization. $CD34^+$ positive cell number was counted in each slice with different five visual fields.

VEC Cell Culture and Grouping

VEC cells were routinely cultured in DMEM medium containing 15% FBS, 1% L-glutamic acid, and 1% penicillin-streptomycin. The cells were maintained at 37°C and 5% CO_2, and passaged at 1:4. The cells in the third generation were used for the following experiments.

Tube formation assay: 200 μl matrigel was added to the 24-well plate and incubated at 37°C for 30 min. VEC cells were seeded in the well at 2×10^5. The VEC cells were divided into four groups. Control group: the cells were resuspended in glucose Earle's balanced salt solution and cultured for 12 h. Then, the cells were changed to DMEM medium and continued culture for 12 h. Oxygen-glucose deprivation (OGD) group: the cells were resuspended in glucose-free Earle's balanced salt solution and further placed in 95% N_2 and 5% CO_2 to prepare the anaerobic environment for 12 h. Then, the cells were changed to DMEM medium and continued culture for 12 h. OGD + G-CSF group: the cells were resuspended in glucose-free Earle's balanced salt solution and further placed in 95% N_2 and 5% CO_2 to prepare the anaerobic environment for 12 h. Then, the cells were changed to DMEM medium containing 10 ng/ml G-CSF and continued culture for 12 h. OGD + G-CSF+LY294002 group: the cells were resuspended in glucose-free Earle's balanced salt solution and further placed in 95% N_2 and 5% CO_2 to prepare the anaerobic environment for 12 h. Finally, the cells were changed to DMEM medium containing 10 ng/ml G-CSF and 10 μM LY294002 and continued culture for 12 h.

RN-c Cell Culture, OGD Treatment, and Grouping

Rat cerebral RN-c cells were routinely cultured in Neurobasal Medium containing 1 ml 2% B27 and 1% GlutaMAX. The cells were maintained at 37°C and 5% CO_2, and passaged at 1:4. The cells in logarithmic phase were used for the following experiments.

The RN-c cells were divided into the above-mentioned four groups and collected after 24 h to test mRNA, protein, and apoptosis.

Flow Cytometry

RN-c cells were digested and washed with cold PBS. After suspended in 500 μl Binding Buffer, the cells were further added with 5 μl Annexin V-FITC and incubated at room temperature for 15 min. Then, the cells were added with 5 μl propidium iodide (PI) and incubated at room temperature for 5 min. At last, the cells were tested on flow cytometry.

Table I. Garcia score in different time points (mean ± SD).

Group	Postoperative 24 h	Postoperative 48 h	Postoperative 72 h
Sham (n=10)	17.23±0.67	17.41±0.71	17.36±0.66
ICH (n=10)	7.61±0.26[a]	7.22±0.25[a]	6.77±0.19[a]
ICH+G-CSF (n=10)	8.77±0.29[b]	9.58±0.33[b]	10.39±0.36[b]
ICH+G-CSF+ LY294002 (n=10)	7.83±0.27[c]	7.87±0.26[c]	8.17±0.28[c]

[a]$p<0.05$, compared with sham group. [b]$p<0.05$, compared with ICH group. [c]$p<0.05$, compared with ICH+G-CSF group.

qRT-PCR

The tissue or cells were treated with TRIzol and extracted using chloroform. The sample was moved to a new Eppendorf (EP) tube and the RNA was obtained after isopropanol sediment, 70% ethanol washing, and DEPC water dissolution. QuantiTect SYBR Green RT-PCR Kit was used to test gene expression. The 20 µl qRT-PCR reaction system contained 10.0 µl 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 µl 0.5 µmol/l primers, 2 µg template RNA, 0.5 µl QuantiTect RT Mix, and ddH_2O. The reverse transcription was performed at 50°C for 30 min. The PCR reaction was performed at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Western Blot Assay

Total protein was extracted by RIPA from cells or tissues. After lysed on ice for 20 min, the sample was centrifuged at 10000 ×g for 10 min. After quantified by BCA method, a total of 40 µg protein was separated by 6%-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane at 100 V for 120 min. Next, the membrane was blocked by 5% skim milk and incubated in primary antibody at 4°C overnight (AKT, p-AKT, Bcl-2, and β-actin at 1:3000, 1:1000, 1:3000, and 1:10000, respectively). Then, the membrane was incubated in HRP labeled secondary antibody (1:25000) for 60 min after washed by PBS Tween-20 (PBST) for three times. At last, the protein expression was detected by ECL chemiluminescence (Amersham Biosciences (Piscataway, NJ, USA).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test. $p<0.05$ was considered as statistical significance.

Results

Neurological Dysfunction Significantly in ICH Model, G-CSF Alleviated Neurological Injury

Garcia method showed that the neurological dysfunction score in ICH group significantly decreased compared with sham group with time dependence ($p<0.05$). G-CSF treatment significantly improved Garcia score compared with that in ICH group ($p<0.05$). The LY294002 treatment markedly attenuated the therapeutic effect of G-CSF on neurological dysfunction ($p<0.05$) (Table I).

LY294002 Significantly Weakened the Neuron Apoptosis Protective Effect and Angiogenesis Promotion of G-CSF

TUNEL assay revealed that cell apoptosis number markedly elevated in the ICH group compared with sham group ($p<0.05$). G-CSF treatment apparently reduced cell apoptosis number compared with ICH group ($p<0.05$). The LY294002 intervention significantly alleviated the protective effect of G-CSF on model rat cerebral neuron apoptosis ($p<0.05$) (Figure 1A).

$CD34^+$ cell number increased in ICH group compared with sham group ($p<0.05$). G-CSF treatment apparently elevated $CD34^+$ cell number compared with ICH group ($p<0.05$). The LY294002 intervention markedly decreased $CD34^+$ cell number compared with G-CSF group ($p<0.05$) (Figure 1B).

G-CSF Activated PI3K/AKT Signaling Pathway in Brain Tissue

Quantitative RT-PCR (qRT-PCR) detection showed that Bcl-2 mRNA in brain tissue significantly reduced, while VEGF mRNA in cerebrovascular intima significantly up-regulated in ICH group compared with sham group, suggesting that Bcl-2 reduction may be related to neuron apoptosis, whereas VEGF over-expression may promote

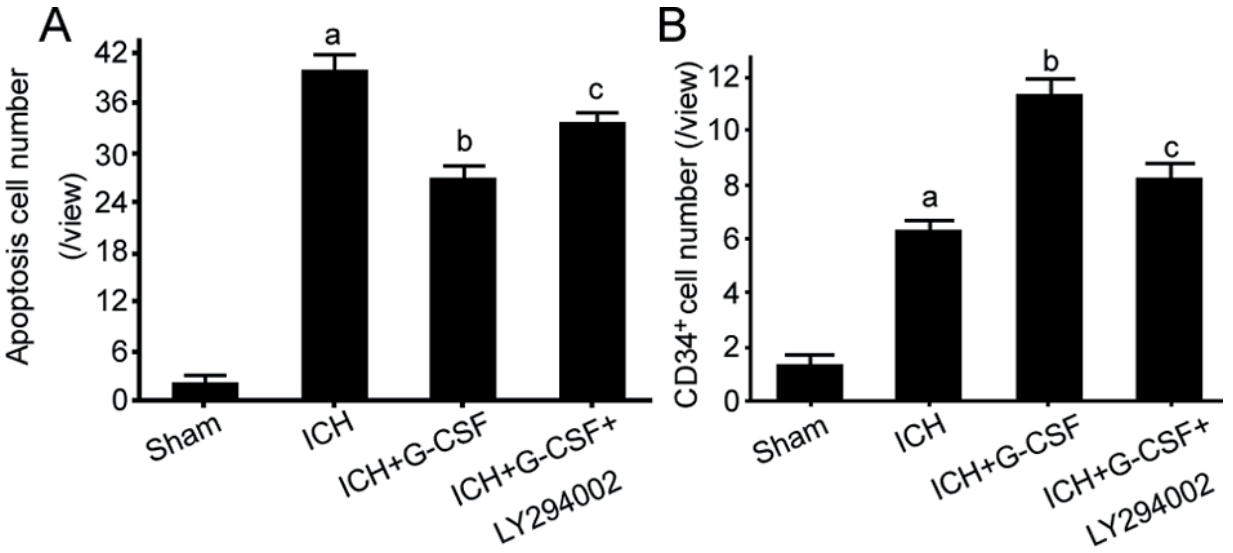

Figure 1. LY294002 significantly weakened the neuron apoptosis protective effect and angiogenesis promotion of G-CSF. ***(A)*** TUNEL assay detection of neuron apoptosis. ***(B)*** CD34 detection of cerebral tissue angiogenesis. [a]$p<0.05$, compared with sham group. [b]$p<0.05$, compared with ICH group. [c]$p<0.05$, compared with ICH+G-CSF group.

angiogenesis. G-CSF treatment markedly elevated Bcl-2 and VEGF mRNA expressions, whereas LY294002 combined intervention attenuated the up-regulatory effect of G-CSF on Bcl-2 and VEGF mRNAs (Figure 2A).

Western blot demonstrated that AKT, p-AKT, and Bcl-2 protein levels were lower, while VEGF protein was markedly higher in the brain tissue and cerebrovascular intima from ICH group than that from sham group. G-CSF activated PI3K/AKT signaling pathway, up-regulated Bcl-2 expression, and facilitated VEGF level in the intima. LY294002 treatment apparently alleviated PI3K/AKT signaling pathway activity, Bcl-2, and VEGF protein expressions (Figure 2B).

G-CSF Reduced Neuron Apoptosis Induced by OGD Through Activating PI3K/AKT Signaling Pathway

Flow cytometry detection revealed that RN-c cell apoptosis significantly enhanced in OGD group compared with control. G-CSF treatment declined cell apoptosis, while LY294002 weakened the G-CSF protective effect on neuron apoptosis (Figure 3A). qRT-PCR demonstrated that OGD markedly down-regulated Bcl-2 mRNA level in RN-c cells. G-CSF attenuated the reduction of Bcl-2 mRNA induced by OGD, while LY294002 alleviated G-CSF induced Bcl-2 mRNA over-expression (Figure 3B). Western blot exhibited that OGD significantly decreased p-AKT and Bcl-2 protein levels in RN-c cells. G-CSF attenuated the reduction of p-AKT and Bcl-2 protein levels induced by OGD, while LY294002 alleviated G-CSF induced p-AKT and Bcl-2 protein over-expression (Figure 3C).

G-CSF Promoted Angiogenesis Through Activating PI3K/AKT Signaling Pathway and VEGF Expression

The qRT-PCR demonstrated that OGD significantly upregulated VEGF mRNA expression in VEC cells. G-CSF further enhanced VEGF mRNA expression based on OGD, while LY294002 alleviated G-CSF induced VEGF mRNA up-regulation (Figure 4A). Western blot exhibited that OGD apparently decreased p-AKT and enhanced VEGF protein levels in VEC cells, indicating that G-CSF plays a role in enhancing AKT phosphorylation and up-regulating VEGF protein. LY294002 alleviated p-AKT and VEGF up-regulation induced by G-CSF (Figure 4B). Tube formation assay revealed that the tube formation ability of VEC cells significantly enhanced in OGD group, and G-CSF treatment further increased tube formation ability induced by OGD, whereas LY294002 restrained the tube formation promotion induced by G-CSF (Figure 4C).

Discussion

Cerebral stroke, also known as cerebrovascular accident (CVA), is a group of acute cerebrovascu-

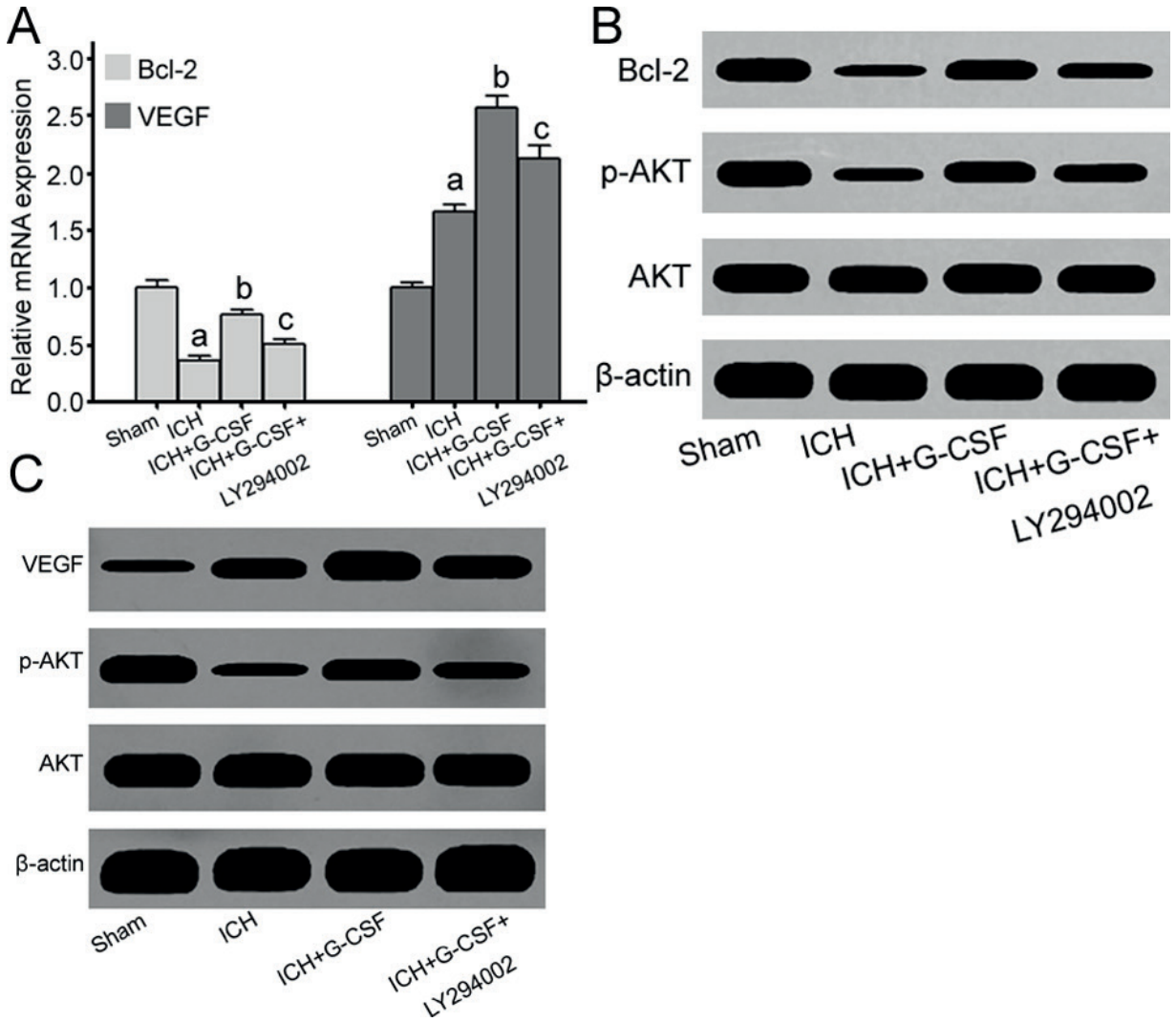

Figure 2. G-CSF activated PI3K/AKT signaling pathway in brain tissue. ***(A)*** qRT-PCR detection of gene expression. ***(B)*** Western blot detection of p-AKT and Bcl-2 protein expressions in brain tissue. ***(C)*** Western blot detection of p-AKT and VEGF protein expressions in cerebrovascular intima. [a]$p<0.05$, compared with sham group. [b]$p<0.05$, compared with ICH group. [c]$p<0.05$, compared with ICH+G-CSF group.

lar disease that caused by ischemic brain tissue damage due to sudden rupture of the blood vessels or angiemphraxis, including cerebral arterial thrombosis and hemorrhagic apoplexy. ICH is the most destructive type of stroke that accounts for 10-15% of all strokes in Western countries. It accounts for 30-35% in China and other Asian countries[3,16,17]. ICH can lead to severe cerebral hematoma, peripheral tissue edema, and secondary nerve cell injury tissue defects. At present, there are no other effective intervention and treatment measures in clinic in addition to surgical removal of hematoma, ease brain edema, and reducing intracranial pressure[18-20]. Apoptosis is one of the most important mechanisms of neurodegeneration caused by ICH. It is a hot issue in ICH research. It is also a measure to reduce the apoptosis and improve the neurological function for the treatment of ICH[4,5].

PI3K is an important member of the growth factor receptor superfamily signal transduction process, which can be activated under the stimulation of a variety of cytokines and mitogen. It activates AKT through a series of intermediate molecules to participate in the regulation of cell survival, proliferation, cycle, and apoptosis[21,22]. PI3K can be activated through conformation change by growth factors, mitogen, and other factors, and promote the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phospha-

tidylinositol (3,4,5)-trisphosphate (PIP3), which can phosphorylate AKT on Ser473 and Thr308 with the help of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. Phosphorylation-activated AKT could control of a variety of target gene transcription and translation processes to regulate cell survival, proliferation, and apoptosis. Bcl-2 is an important anti-apoptotic factor that can affect mitochondrial function, inhibit the release of cytochrome C (Cyt C), and affect the trans-membrane transport of calcium, and suppress apoptotic protease activating factor-1 (Apaf-1) activation[23].

Our results showed that compared with Sham group, the neurological dysfunction score was significantly reduced in ICH group, indicating successful ICH modeling. Guo et al[7] revealed that compared with the untreated group, G-CSF treatment obviously accelerated the recovery of brain function in ICH rats and activated the proliferation of nerve cells. Chu et al[24] found that G-CSF treatment markedly improved the brain function of ICH mice. In this study, the neurological deficits of ICH rats were apparently improved after G-CSF treatment, which was similar to Guo et al[7] and Chu et al[24]. Our research observed that compared with Sham group, brain neuronal apoptosis significantly increased in the rat from ICH model, while G-CSF intervention significantly reduced the brain nerve cell apoptosis, revealing that apoptosis may play a role in the process of ICH and G-CSF may protect and improve brain function after ICH by reducing apoptosis. Inhibition of the transcriptional activity of PI3K/AKT signaling pathway significantly weakened the therapeutic effect of G-CSF[7,24]. G-CSF treatment markedly reduced the apoptosis of rat brain cells after ICH, which was consistent with the reduced impact of G-CSF on cerebral neuronal apoptosis after ICH. $CD34^+$ cell counting demonstrated that the angiogenesis was significantly increased in the brain tissue of ICH model, suggesting it may be a compensatory protective mecha-

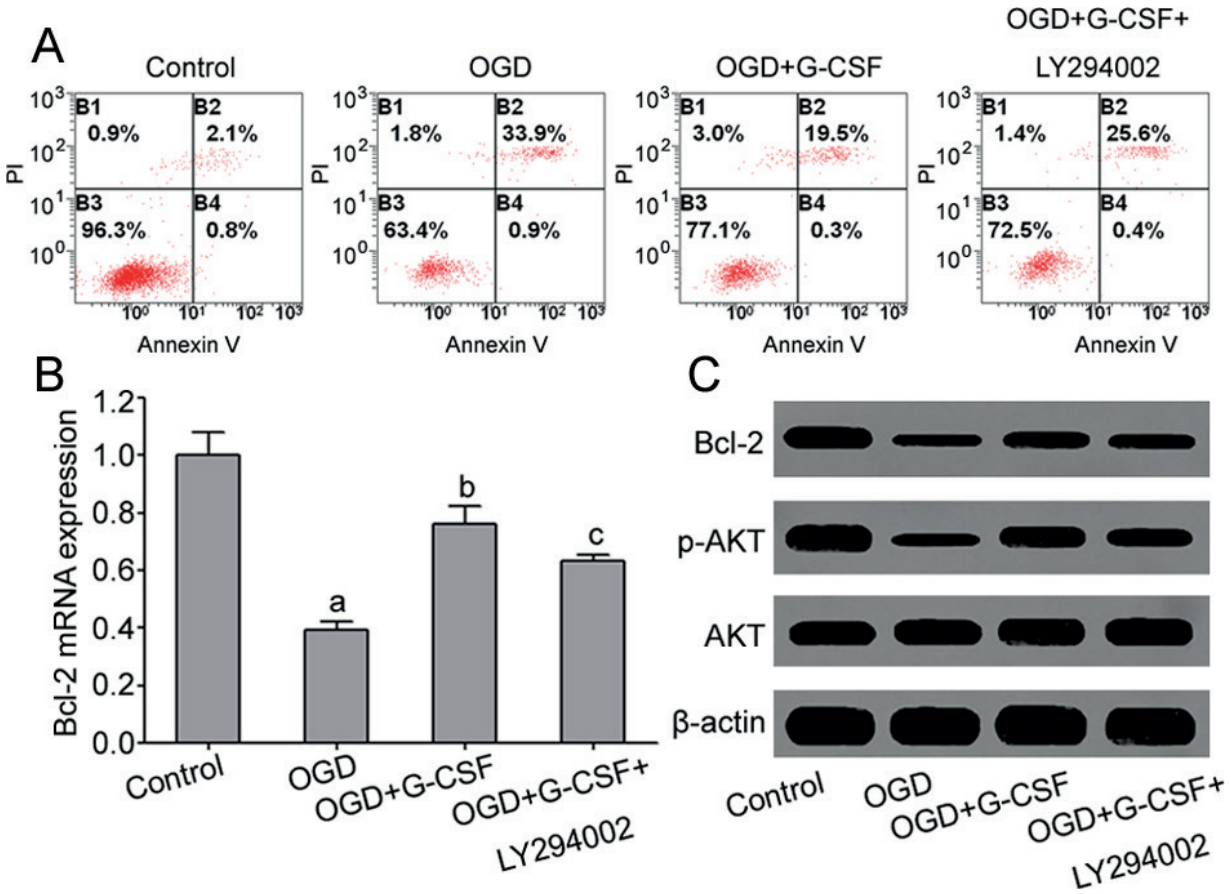

Figure 3. G-CSF reduced neuron apoptosis induced by OGD through activating PI3K/AKT signaling pathway. (***A)*** Flow cytometry detection of cell apoptosis. ***(B)*** qRT-PCR detection of mRNA expression. (***C)*** Western blot detection of protein expression. [a]$p<0.05$, compared with control group. [b]$p<0.05$, compared with OGD group. [c]$p<0.05$, compared with OGD+G-CSF group.

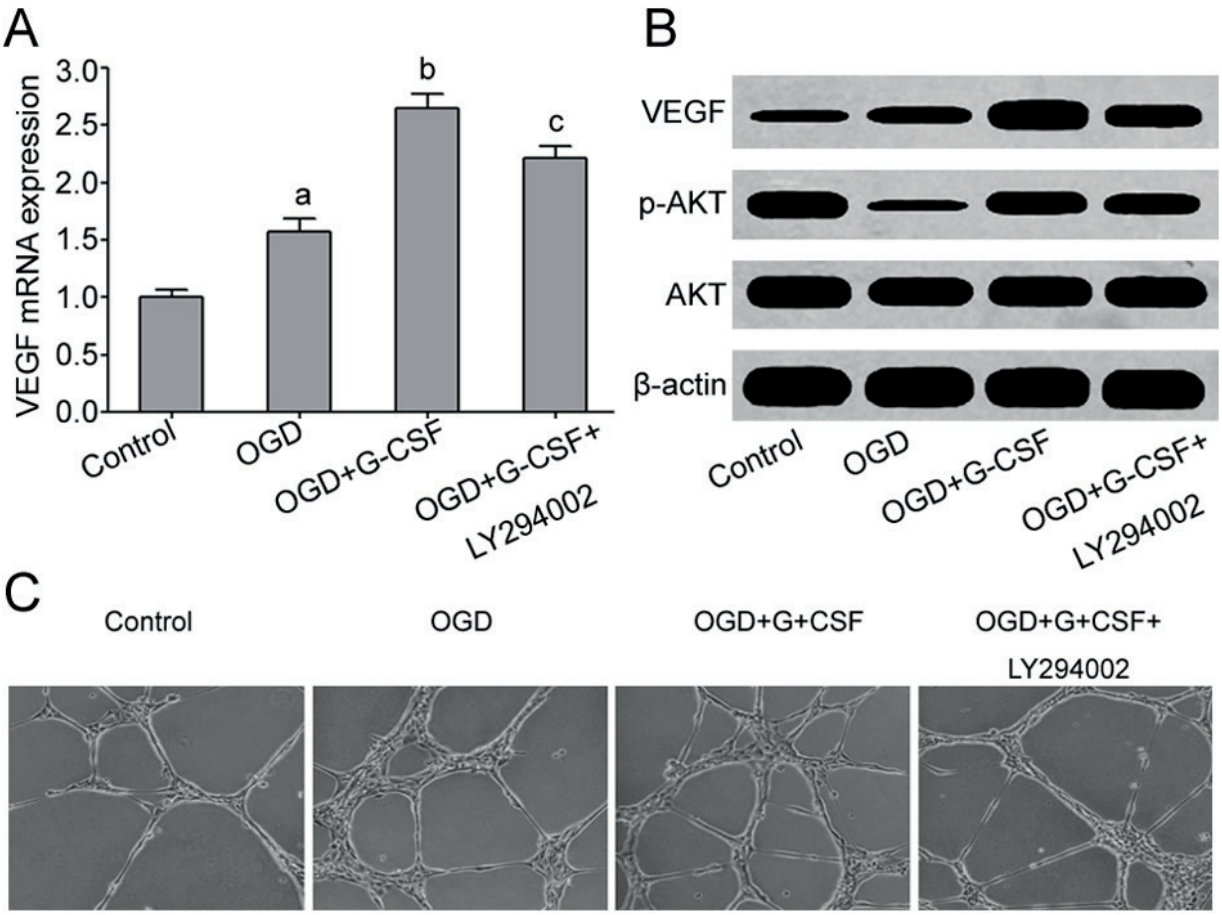

Figure 4. G-CSF promoted angiogenesis through activating PI3K/AKT signaling pathway and VEGF expression. ***(A)*** qRT-PCR detection of mRNA expression. ***(B)*** Western blot detection of protein expression. ***(C)*** Tube formation assay detection of tube formation ability in VEC cells. [a]$p<0.05$, compared with control group. [b]$p<0.05$, compared with OGD group. [c]$p<0.05$, compared with OGD+G-CSF group.

nism. G-CSF further enhanced angiogenesis, while LY294002 restrained angiogenesis induced by G-CSF. Duelsner et al[25] reported that G-CSF significantly promoted the growth of collateral vessels and improved the reserve capacity of cerebrovascular vessels in cerebrovascular accident animal model. The results of mRNA and protein further demonstrated that G-CSF enhanced the activity of PI3K/AKT pathway, increase the expression of anti-apoptotic factor Bcl-2 in brain tissue of ICH rats, and elevated the expression of VEGF in the endometrium. LY294002 reduced Bcl-2 and VEGF levels on the basis of G-CSF therapy. Our results exhibited that activation of PI3K/AKT pathway, up-regulation of Bcl-2 and VEGF expressions were the mechanism of G-CSF in protecting nerve cells and promoting angiogenesis. Previous studies suggested that the expression levels of VEGF and VEGF receptors in the brain tissue around the hematoma of the ICH mice after G-CSF treatment were significantly higher than those in the untreated group, which was mediated by activation of the ERK and STAT3 signaling pathways[24]. We observed the impact of G-CSF in upregulating VEGF expression in brain tissue. We found that G-CSF treatment significantly improved the neurological dysfunction in ICH model. PI3K/AKT specific inhibitor LY294002 treatment markedly restrained the treatment and improvement effects of G-CSF on neurological dysfunction, leading to a reduced Garcia score. It was showed that G-CSF may play a protective role against brain function by activating PI3K/AKT signaling pathway, while LY294002 can antagonize the protective effect.

Since blood-brain barrier damage, cerebral blood flow obstruction, and brain tissue ischemia are the important pathological basis of neuronal apoptosis after nerve hemorrhage, nerve tissue structure and functional injury, and angiogenesis, this study adopted rat brain nerve cells and va-

scular endothelial cells for OGD to investigate the mechanism of G-CSF on neuronal apoptosis and angiogenesis. The results demonstrated that OGD treatment significantly downregulated PI3K/AKT activity and Bcl-2 expression, induced apoptosis of rat brain neurons, and enhanced the tube formation ability of VEC cells and VEGF expression. G-CSF attenuated the induction influence of OGD on neuronal apoptosis and further enhanced the tube formation ability of VEC cells and VEGF expression. LY294002 antagonized the anti-apoptosis and pro-angiogenesis function of G-CSF. Tsai et al[26] reported that G-CSF protected retinal neurons from apoptosis by activating the PI3K/AKT signaling pathway. Furmento et al[15] suggested that G-CSF promoted VEGF expression in trophoblast Swan 71, which was associated with activation of PI3K/AKT and ERK1/2. Though related researches confirmed that G-CSF can affect the PI3KAKT signaling pathway and regulate VEGF expression, there is still lack of evidence about the regulatory relationship plays a role in the G-CSF treatment of ICH. This study indicated that G-CSF played an important role in the treatment of ICH by enhancing PI3K/AKT signaling pathway, elevating Bcl-2 and VEGF expressions, reducing neuronal apoptosis, and promoting angiogenesis.

Conclusions

We found that G-CSF enhanced PI3K/AKT signaling pathway activity, promoted Bcl-2 and VEGF expression, reduced nerve cell apoptosis, and enhanced tube capacity of VECs, which may be the mechanism of G-CSF in improving neurological function and angiogenesis after ICH.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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