Article

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Study on Neuroprotective Function and Mechanism of 7,8-DHF-Activated Neurotrophic Factor Receptor TrkB in Intracerebral Hemorrhage

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Background: 7,8-dihydroxyflavone (DHF) is a potent agonist of tropomyosin-related kinase B (TrkB), which binds to TrkB and causes TrkB phosphorylation, reducing cell apoptosis improves the stability of the nervous system. It has been shown to play a therapeutic role in various animal disease models, such as ischemic stroke, traumatic brain injury, and Alzheimer's disease. To investigate the protective effect of 7,8-dihydroxyflavone (7,8-DHF) on neuronal cells, we explore the improvement effect of 7,8-DHF on intracerebral hemorrhage (ICH) by activating the TrkB signaling pathway, and study the related mechanism.

Methods: Venous blood samples were collected from patients with ICH before and after treatment and normal people in a fasted state, and mRNA expression levels of brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB), phosphatidylinositol 3 kinases (PI3K), protein kinase B (AKT), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (Erk), and other genes were determined by real-time quantitative PCR (RT-qPCR). An animal model of C57BL/6J mice with ICH was established by injecting collagenase and heparin into the cerebral striatum, with normal C57BL/6J mice as controls. Both groups were injected intraperitoneally or intravenously with DHF or normal saline once a day for two weeks before euthanasia. The neuronal and glial cells of the cerebral cortex striatum were collected from the mice, from which the apoptosis of neuronal and glial cells was detected by ELISA, and mRNA expression of BDNF, TrkB, PI3K, AKT, MAPK, and Erk genes were detected by qPCR.

Results: The RNA expression (BDNF, TrkB, PI3K, AKT, MAPK, and Erk) in the ICH treat group was significantly higher than in the ICH group, without significant difference in RNA (BDNF, TrkB, PI3K, AKT, MAPK, Erk) between ICH treat group and Normal group. Compared with the ICH group, the apoptosis of neuronal and glial cells of ICH mice significantly decreased. At the same time, the expression levels of BDNF, TrkB, PI3K, AKT, MAPK, and Erk were significantly increased. DHF promotes the expression of cellular neurotrophic factor-related genes and proteins in ICH mice and neuronal cell models.

Conclusion: 7,8-DHF can inhibit the apoptosis of neuronal and glial cells, promote their proliferation, increase mRNA expression levels of neurotrophin and its receptors, and activate neurotrophic factor receptors. 7,8-DHF has the potential to protect neuronal cells and possibly improve ICH.

Keywords: 7,8-dihydroxyflavone (DHF); protective effect; neuronal cells; ICH; improvement effect

Introduction

Intracerebral hemorrhage (ICH) is the second most common subtype of stroke, with a high fatality rate and morbidity. Current therapeutic interventions mainly focus on supportive care and surgery. Tropomyosin-related kinase B (TrkB) signaling is recognized as an important protective mechanism for brain injury and a key regulator of neuronal survival [1].

7,8-dihydroxyflavone (7,8-DHF) is a member of the flavonoid family. Flavonoids are a diverse group of plant secondary metabolites present in fruits and vegetables with diverse biological functions, which can act as cancer-preventive agents to protect vulnerable neurons and stimulate neuronal regeneration [2]. 7,8-DHF is a potent neurotrophic factor receptor agonist that binds to TrkB and causes phosphorylation [3]. However, it will not affect the activation of other Trk families and can effectively activate their downstream phosphatidylinositol 3 kinases/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) signaling pathways to combat cellular apoptosis [4]. *In vitro* studies show that 7,8-DHF protects the apoptosis induced by various stimuli (such as glutamate and H_2O_2), and the stimulation of the mechanism is secondary to ICH. 7,8-DHF also can enhance the activation of phosphorylated TrkB in

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the brain [1] and has been shown to play therapeutic roles in various animal disease models, such as ischemic stroke [5], traumatic brain injury [6], and Alzheimer's disease [4]. These conditions have all been associated with insufficient brain-derived neurotrophic factor (BDNF) signaling.

Neurotrophic factors in mammals are composed of four related proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), and neurotrophic factor-4 (NT-4/5). These factors are important regulators of neuron development in vertebrates, mediating neurogenesis, neurosurvival, neurogrowth, and neuroplasticity [7]. BDNF is a non-glycosylated polypeptide of 119 amino acids and has been identified as the second member of the neurotrophin family. Two classes of cell surface receptors mediate the effects of BDNF: the p75 neurotrophic factor receptor (p75NTR) and the TrkB receptor. TrkB-dependent signaling is important in both development and adulthood. The binding of BDNF with the TrkB receptor causes its dimerization and self-phosphorylation. It activates three major signaling pathways: phosphatidylinositol 3 kinases/protein kinase B (PI3K/AKT), mitogen-activated protein kinase (MAPK), and phospholipase C-γ1 pathway [8]. BDNF is a ligand for TrkB that mediates neuron survival, neuronal differentiation, and synaptic plasticity. However, BDNF is not used to treat neurodegenerative diseases due to poor pharmacokinetic characteristics, side effects, and lack of survival characteristics in clinical trials. Therefore, applying alternative methods, such as TrkB receptor agonists, is increasingly important [9]. In this study, we found that the expression of neurotrophic factor gene in human serum was increased after 7,8-DHF treatment. In mice models, 7,8-DHF treatment promotes the proliferative capacity of neuronal and glial cells in injured mice, inhibits apoptosis, and promotes the expression of cellular neurotrophic factor-related genes and proteins. Together, these data suggest that 7,8-DHF can potentially protect neuronal and glial cells and improve ICH.

Nevertheless, the therapeutic effect of 7,8-DHF on ICH has not been determined. The present study aimed to determine whether the TrkB signal pathway is activated by 7,8-DHF and has a protective effect on ICH neurons *in vivo* and *in vitro*. We further investigated whether 7,8-DHF can promote the expression of PI3K/AKT, MAPK, or Erk pathway activation-related molecules downstream of TrkB to infer whether 7,8-DHF is protective against neural cell injury.

Experimental Methods

General Information

Thirty patients (50–80 years old, male or female) with hypertensive ICH at The Second Affiliated Hospital of Qiqihar Medical University were collected. Inclusion criteria for this study were as follows: patients with a history of hypertension who were receiving medical therapy and blood pressure management (systolic blood pressure goal of 140 to 179 mmHg and diastolic blood pressure goal of 70 to 100 mmHg) during hospitalization; patients with the hematoma site located in one of the cerebral hemispheres, with the hematoma volume of 10–30 mL; patients with no blood in ventricles; patients within 24 hours of onset of first acute ICH; patients without loss of consciousness (somnolence is acceptable).

Exclusion criteria for this study included patients with cerebellar or brainstem hemorrhage; a Glasgow Coma Scale (GCS) score between 13 and 15; patients with intracerebral hemorrhage due to bleeding diathesis, aneurysms, vascular malformations, inappropriate use of anticoagulants, or suspected amyloid angiopathy; patients with subarachnoid hemorrhage; patients with multifocal hemorrhage; patients with mixed stroke or hemorrhagic infarction; patients with coexisting systemic diseases such as heart and kidney failure, tumors, gastrointestinal bleeding, etc.; pregnant or lactating women; patients with a history of allergy to Sanqi Panax Notoginseng Injection.

Thirty normal people were collected as controls. Patients with ICH received conventional treatments for 3 days. Conventional treatments included Dehydration therapy with 20% mannitol (National medicine approval number H20184101, Tiansheng Pharmaceutical Group, Chongqing, China) at a dose of 125–250 mL once every 8 h. Besides, treatment of complications was provided as needed, including hypoglycemic therapy for hyperglycemia, antihypertensive therapy for hypertension, anti-inflammatory therapy for infection, acid suppressants for peptic ulcers, and supportive therapies, such as physical cooling, nutritional support, fluid and electrolyte balance. Brain CTs were reviewed on Day 4, and patients in the ICH group were given conventional treatments starting on Day 4 with 14 injections of DHF (7,8-DHF,38183-03-8, Suzhou Mai Lun Biotechnology, Suzhou, China) as the main component, once a day. Venous blood samples were collected in a fasted state before and after treatment. Collection occurred at 8–9 a.m. at admission before treatment and at 8–9 a.m. after 14 days post-treatment. The blood was collected from the normal control group at the same time. The normal control patients had no history of cerebral hemorrhage and no hypertension. The study was approved by the Review Committee and reviewed by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University ((Qi) Ethics Review [2019] No. 1201), and all the patients involved in the project signed the informed consent forms. The study was carried out in accordance with the Helsinki Declaration.

Cell Lines and Cell Cultures

Neuronal cell lines of mice, HT22 (CL-0697, Wuhan Pricella Biotechnology, Wuhan, China) and NSC-34 (409-21-2, ScienCell, Beijing, China), Growth medium, MEM medium (41500034, Gibco, Big Island, NY, USA) + 10%FBS (A5669701, Gibco, Big Island, NY, USA) +

Table 1. Bederson scoring [10] criteria.

Score (points)	Scoring Criteria
0	No neurological deficits
1	Any flexion component in the forelimbs, that is, positive results in the tail lift test, without other abnormalities
2	Decreased resistance to lateral push, that is, positive results in the lateral push test, with forelimb flexion but without turning behavior
3	Behaviors as described above with a score of 2, with spontaneous rotations, marking circles toward the paralyzed side during free movement

1%P/S (15140163, Gibco, Big Island, NY, USA). After 85-90% fusion, the cells were passaged, digested with 0.25% pancreatin (25200072, Gibco, Big Island, NY, USA) for 2 min, and digested with medium containing 10% FBS. Washed cells were collected and sub-cultured by 1:2–1:3. The medium was changed 2–3 times a week, with the concentration of carbon dioxide in the carbon dioxide incubator of 5% and the temperature of 37 °C. Cells were validated by STR and verified negative for mycoplasma.

Establishment of a Mouse Model of ICH

60 adult female C57BL/6J mice, 6 weeks of age, each weighing 20 g, were purchased and allocated to six groups with 10 mice per group. These include CH mouse + 0.9% NaCl group, CH mouse + DHF (7,8-DHF, 38183-03-8, Suzhou Mai Lun Biotechnology, Suzhou, China) group (20 mg-1W), CH mouse + DHF group (20 mg-2W), CH mouse + DHF group (40 mg-1W), and CH mouse + DHF group (40 mg-2W) and Normal mouse + 0.9% NaCl group. Adult female C57BL/6J mice (hnslkjd005, Slack Jingda, Hunan, China) were anesthetized with 3% sodium pentobarbital (Sinopiate number H31021724, Shanghai Shangyao Xinya Pharmaceutical, Shanghai, China) by intraperitoneal injection at 10 mL/kg. The anesthetized mice were fixed prone (back-up) on plates with a rope, tied with a slipknot for the rope so it wouldn't slip. During fixation, the mouse's two front and two back feet were kept in a straight line, respectively, and the mouse's head was slightly raised with cotton to facilitate cutting the skin. The mouse's head was cut about 3~5 cm along the miter slit to reveal the skull. Marks at 1.5 cm to the anterior halo and 2.5 cm to the left next to the midline were made and drilled perpendicularly at the mark with a borer to a depth that should not be too large to stop when there is a duty off to prevent drilling into brain tissues. The needle tube was inserted into the cranial cavity of mice with an insertion depth of approximately 1.5 cm, 0.5 μL of 1.0 μg/μL VII collagenase (17101015, Gibco, Big Island, NY, USA), and 1.0 μL (7U) of sodium heparin (R21525, Shanghai Yuanye Biotechnology, Shanghai, China) were injected to make a mouse striatal hemorrhage model. The head skin of the mice was re-sutured with a suture needle, and after suture, the mice were removed from the rat board and placed back into the cage for rest. At 24 h after model preparation, mice with scores above 3 were selected according to the Bederson scoring criteria for subsequent experiments (Table 1, Ref. [10]). All animal experiments were approved by the Ethical Committee of the Second Affiliated Hospital of Qiqihar Medical University ((Qi) Ethics Review [2019] No. 1201).

Establishment of a Neuronal Cell Injury Model

Neuronal cells of mice, HT22 and NSC-34, were resuscitated and cultured; the cells showed epithelial growth, then imaged based on anti-beta III Tubulin monoclonal and secondary antibody staining, respectively. They were used to determine the correct cell type before the experiment began. Cells were then inoculated into 6-well plates with a density of 8000 cells/cm^2. Hemin (10 μM Hemin, 51280, Sigma, St. Louis, MO, USA) was added into the medium, with the concentration of carbon dioxide in the carbon dioxide incubator of 5% and the temperature of 37 °C, for 12 h. Subsequent experiments were conducted to construct a neuronal cell injury model induced by Hemin [11].

qPCR

The models of neuronal cell injury induced by Hemin in mice's neurons (HT22 and NSC-34) were divided into four groups. In the CH cell + 5 mM DHF group, 5mM DHF was added to injured neuronal cells. CH cell + 10 mM DHF group was the control group, in which an equal amount of saline was added to injured neuronal cells. In the CH cell + 0.9% NaCl group, an equal amount of saline was added to normal neuronal cells. All the above operations lasted for 24 h. Serum was extracted from the patient's blood samples at 3000 rpm/min, and total RNA was extracted from the patient's serum samples, neuronal cells of mice (HT22 and NSC-34), primary neuronal and glial cells of mice, etc., with TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA). RNA (100 ng) was reverse transcribed to cDNA using the TaqMan reverse transcription Kit (N8080234, Invitrogen, Carlsbad, CA, USA). qPCR was performed with the real-time PCR amplification kit (A46113, Applied Biosystems^{TM}, Carlsbad, CA, USA) to amplify BDNF, TrkB, PI3K, AKT, MAPK, ERK and calculate relative expression levels. All the experiments were performed in triplicate. Primer sequences and internal references are shown in Table 2. The relative quantitative results were calculated using the method of $2^{-\Delta\Delta Ct}$ [12].

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Table 2. Primer sequences and internal references (mice).

Genes	Primers	Sequences
BDNF	Forward	5′-TCATACTTCGGTTGCATGAAGG-3′
	Reverse	5′-ACACCTGGGTAGGCCAAGTT-3′
TrkB	Forward	5′-CTGGGGCTTATGCCTGCTG-3′
	Reverse	5′-AGGCTCAGTACACCAAATCCTA-3′
PI3K	Forward	5′-CGAGAGTGTCGTCACAGTGTC-3′
	Reverse	5′-TGTTCGCTTCCACAAACACAG-3′
AKT	Forward	5′-CCCTGCTCCTAGTCCACCA-3′
	Reverse	5′-TGTCTCTGTTTCAGTGGGCTC-3′
MAPK	Forward	5′-GCTTTGACGCAGGTGCTAAG-3′
	Reverse	5′-TGTCCCCATAACCGGAGTAGG-3′
Erk	Forward	5′-TCCACGTCTATTACGAGAAGGT-3′
	Reverse	5′-CAACACCATCAAATCCTCGGAG-3′
GAPDH	Forward	5′-GGGCTGCTTTTAACTCTGGT-3′
	Reverse	5′-TGATTTTGGAGGGATCTCGC-3′

BDNF, brain-derived neurotrophic factor; *TrkB*, tropomyosin-related kinase B; *PI3K*, phosphatidylinositol 3 kinases; *AKT*, protein kinase B; *MAPK*, mitogen-activated protein kinase; *Erk*, extracellular signal-regulated kinase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Primer sequences and internal references (human).

Genes	Primers	Sequences
BDNF	Forward	5′-GGCTTGACATCATTGGCTGAC-3′
	Reverse	5′-CATTGGGCCGAACTTTCTGGT-3′
TrkB	Forward	5′-TCGTGGCATTTCCGAGATTGG-3′
	Reverse	5′-TCGTCAGTTTGTTTCGGGTAAA-3′
PI3K	Forward	5′-TATTTGGACTTTGCGACAAGACT-3′
	Reverse	5′-TCGAACGTACTGGTCTGGATAG-3′
AKT	Forward	5′-AGCGACGTGGCTATTGTGAAG-3′
	Reverse	5′-GCCATCATTCTTGAGGAGGAAGT-3′
MAPK	Forward	5′-TACACCAACCTCTCGTACATCG-3′
	Reverse	5′-CATGTCTGAAGCGCAGTAAGATT-3′
Erk	Forward	5′-TCCACGTCTATTACGAGAAGGT-3′
	Reverse	5′-ATGCAGCCTACAGACCAAATATC-3′
GAPDH	Forward	5′-GGAGCGAGATCCCTCCAAAAT-3′
	Reverse	5′-GGCTGTTGTCATACTTCTCATGG-3′

Venous blood samples were collected from patients with ICH (30 patients, ICH group) and normal population (30 patients, Normal group) in fasted state before treatment (ICH group) and after treatment (ICH treat group) separately. Venous blood samples were collected from controls under fasting conditions at the same time point. mRNA levels of BDNF, TrkB, PI3K, AKT, MAPK, and Erk were analyzed for the differences in mRNA levels and mRNA levels before and after treatment of ICH patients and normal controls. qPCR was performed with the real-time PCR amplification kit (A46113, Applied Biosystems^{TM}, Carlsbad, CA, USA) to amplify BDNF, TrkB, PI3K, AKT, MAPK, ERK and calculate relative expression levels. All the experiments were performed in triplicate. The data were statistically analyzed by software (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA, USA). Primer sequences and internal references are shown in Table 3.

WB

The models of neuronal cell injury induced by Hemin in mice's neurons (HT22 and NSC-34) were divided into four groups. In the CH cell + 5 mM DHF group, 5 mM DHF was added to injured neuronal cells. CH cell + 10 mM DHF group was the control group, in which an equal amount of saline was added to injured neuronal cells. In the CH cell + 0.9% NaCl group, an equal amount of saline was added to normal neuronal cells. All the above operations lasted for 24 h. Cells were lysed in precooled RIPA lysate with 1% PMSF (L00025, Beyotime, Shanghai, China), incubated on ice for 30 min, and then centrifuged for 10 min (12,000 ×g, at 4 °C). The supernatant was collected, and the protein concentration was determined using the Pierce BCA Protein Assay Kit (23227, Thermo Scientific, Pudong New District, Shanghai, China). Protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membrane (88585, Thermo Scientific, Pudong New District, Shanghai, China). PVDF membrane was blocked in TBST buffer containing 5% defatted milk powder for 2 h and incubated with TrkB (ab187041, Abcam, Cambridge, MA, USA), p-TrKB (1:5000, ab229908, Abcam, Cambridge, MA, USA), Bcl2 (1:5000, ab182858, Abcam, Cambridge, MA, USA), Bax (1:5000, ab32503, Abcam, Cambridge, MA, USA) and β-actin (1:5000, ab8226, Abcam, Cambridge, MA, USA) antibodies at 4 °C overnight. Horseradish peroxidase-conjugated antibody (1:2000, 20758, Proteintech, Wuhan, China) was used for 1 h secondary antibody incubation at room temperature. Finally, the target protein bands were detected using the ECL detection system (P0018S, Beyotime, Shanghai, China). Protein detection module in chemiluminescence image analysis system (Tanon 5200, Tanon, Shanghai, China) was used to collect luminescence results.

Lactate Dehydrogenase (LDH) for Apoptosis

Cells were divided into 4 groups: CH cell + 5mM DHF (7,8-DHF, 38183-03-8, Suzhou Mai Lun Biotechnology, Suzhou, China) group, in which 5 mM of DHF was added in injured neuronal cells, CH cell + 10 mM DHF group (DHF treat group with 10 mM), in which 10 mM of DHF was added in injured neuronal cells, CH cell + 0.9% NaCl group (control group), in which an equal amount of saline was added in injured neuronal cells, and Normal cell + 0.9% NaCl group, in which an equal amount of saline was added in injured neuronal cells, and the apoptosis of neuronal cells was detected by LDH (C0016, Beyotime, Shanghai, China) at 12 h and 24 h, respectively. Neuronal cells were collected and counted, with a cell density of 1 ×

10^6 cells/mL; 100 µL of cells were inoculated to 96-well plates per well, which were then divided into control wells, experimental wells, and maximum enzymatic live control wells. The cells were cultured with MEM medium + 10% FBS + 1% P/S for 12 h and tested at different time points of the experimental setup. 30 min before detection at each time point, 10 µL of LDH-releasing reagent was added into the control well with maximum enzyme activity. After repeated blowing, the reagent was mixed and then incubated in the cell incubator for 30 min. During the test, 100 µL of LDH test working solution was added to each well, mixed, incubated at room temperature, and kept away from light for about 30 min; then 50 µL of termination solution was added to each well to terminate the reaction. The OD of each group was measured at 490 nm and calculated, and the data was then subtracted from the control hole after the background blank.

Apoptosis rate = (OD of treated sample – Control OD of sample) / (OD of maximum enzyme activity of cells – OD of sample control well)

C57BL/6J mouse ICH model was divided into four groups: CH cell + 5 mM DHF group, CH cell + 10 mM DHF group, CH cell + 0.9% NaCl group and Normal cell + 0.9% NaCl group, among which CH cell + 5 mM DHF group and CH cell + 10 mM DHF group were model groups and CH cell + 0.9% NaCl group was consisted of normal mice as controls, with ten mice in each group. CH cell + 5 mM DHF group underwent an intraperitoneal injection of 40 mg/kg DHF. CH cell + 10 mM DHF group was the control group, and an equivalent amount of normal saline injection was injected intraperitoneally. In the CH cell + 0.9% NaCl group, normal C57BL/6J mice were injected intraperitoneally with equivalent amounts of normal saline. All the operations were conducted once a day for 2 weeks. After the last injection, the mice were euthanized after 24 h by intraperitoneal injection of pentobarbital sodium (30 mg/kg), 0.1 mL per mouse. After the mice were severely anesthetized, the scalp and skull were cut, and the brain tissues were removed and placed in a cold plate containing pH 7.2 and D-Hank's solution without calcium and magnesium. The hippocampal tissues were separated aseptically. With the back of the brain tissues positioned upward, the cerebral cortex was carefully open under the mirror and the hippocampus was exposed. The tissues around the hippocampus were separated with ophthalmic scissors or sharp forceps, then removed and put into a plate containing D-Hank's solution. Subsequently, the tissues were cut into about 1 mm^3 tissue blocks and ground with rubber tip of disposable syringe. After that, the tissues were suspended with a medium (MEM medium + 10% FBS + 1% P/S) and gently blown with a straw 20 times to make cell suspension. Cell suspensions are filtered with 200 mesh stainless steel mesh. Neuronal and glial cells were extracted to detect apoptosis or other indicators. Cell STR was identified as qualified. The results of the procured cell line mycoplasma were negative. Neuronal and glial cells of mice were resuscitated and cultured, showing epithelial growth. Subsequently, the cells were imaged based on anti-beta III Tubulin monoclonal (ab18207, Abcam, Cambridge, UK) and secondary antibody (A0562, Beyotime, Shanghai, China) staining, respectively.

Cell Counting Kit-8 (CCK-8) for Cell Proliferation

The models of neuronal cell injury induced by Hemin in mice's neurons (HT22 and NSC-34) were divided into four groups. In the CH cell + 5 mM DHF group, 5 mM DHF was added to injured neuronal cells. CH cell + 10 mM DHF group was the control group, in which an equal amount of saline was added to injured neuronal cells. In the CH cell + 0.9% NaCl group, an equal amount of saline was added to normal neuronal cells. All the above operations lasted for 24 h. 100 µL of (3E4 cells/mL) cell suspension was added to 96-well plates. The plates were pre-incubated in the incubator for 24 h (under the conditions at 37 °C and 5% CO_2). 10 µL of the substance at different concentrations was added to be tested to the plates and incubated in the incubator for 24 h. 10 µL of CCK-8 (C0038, Beyotime, Shanghai, China) solution was added to each well carefully without producing bubbles in the hole, so as not to affect the reading of OD value. The plates were incubated in the incubator for 1–4 h, and OD at 450 nm was measured with an enzyme label (Fluoroskan™, Product code: 5200110, Thermo Scientific, Pudong New District, Shanghai, China). The absorbance values on 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, and 7 d were detected, respectively, and the growth curve of cells was drawn (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA, USA).

Statistical Analysis

Data analysis was performed with SPSS software (IBM SPSS Statistics version 22, IBM SPSS Inc., Chicago, IL, USA). Between-group comparisons were performed using *t*-test or paired *t*-test, while comparison among multiple groups utilized one-way ANOVA with SNK-q test for pairwise comparisons. A two-tailed *p*-value less than 0.05 was considered statistically significant. Asterisks indicated significant degree: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Measurement variables following a normal distribution are presented as mean and standard deviation ($\bar{x} \pm s$). Pearson's correlation analysis was employed to investigate the association between two normally distributed quantitative variables. All tests were biologically repeated 3 times, and abnormal data beyond the inclusion or exclusion criteria were excluded during data analysis.

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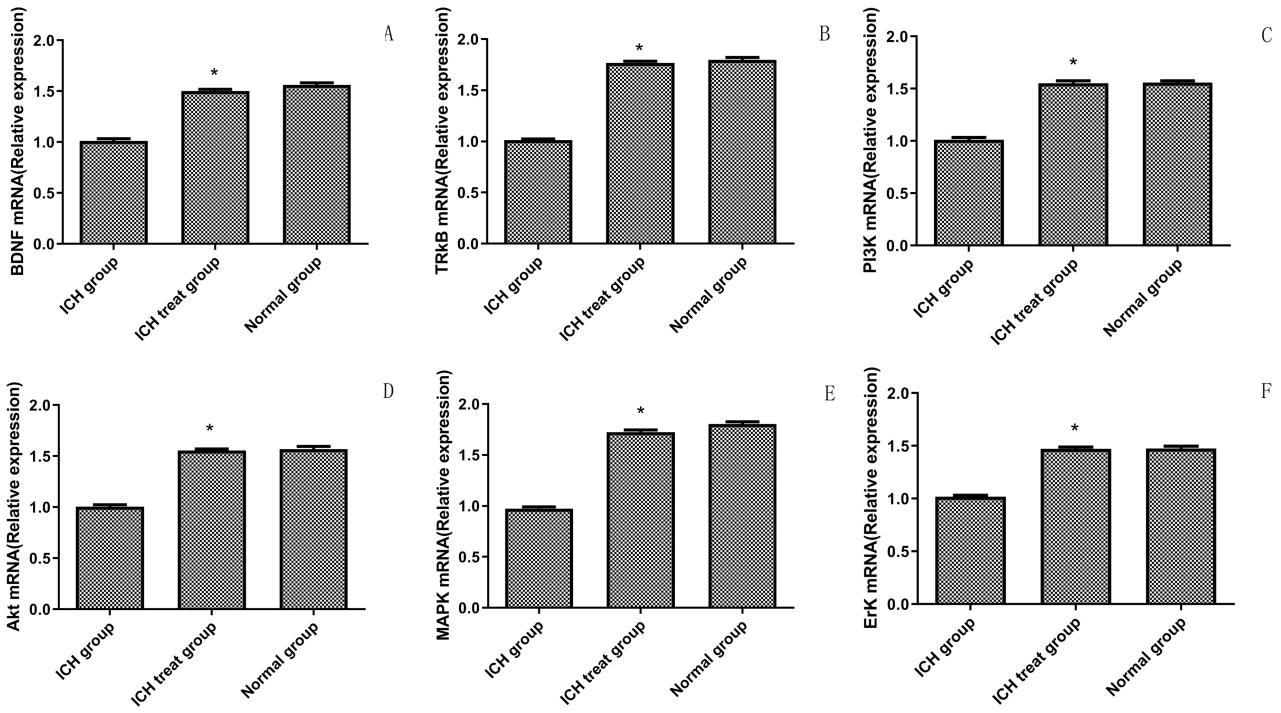

Fig. 1. The expression of the neurotrophic factor gene in human serum was increased after DHF treatment. Relative gene expression levels of (A) BDNF, (B) TrkB, (C) PI3K, (D) AKT, (E) MAPK, and (F) Erk. ($p > 0.05$, n = 30). * represents significant differences between the two groups ($p < 0.05$).

Results

The Expression of the Neurotrophic Factor Gene in Human Serum was Increased after DHF Treatment

The results showed that RNA expression (BDNF, TrkB, PI3K, AKT, MAPK, and Erk) was significantly higher in the ICH treat group than that in the ICH group, without significant difference in RNA (BDNF, TrkB, PI3K, AKT, MAPK, Erk) between ICH treat group and Normal group. See Fig. 1.

DHF Inhibits the Apoptosis of Neuronal Cells in Vitro

The mice's embryonic neurons were used to construct a Hemin-induced neuronal cell injury model. The results showed that after HT22 was exposed to DHF for 12 h, the apoptosis rate in CH cell + 10 mM DHF group was significantly lower than that in CH cell + 5 mM DHF group ($p < 0.05$) and that in CH cell + 10 mM DHF group was significantly lower in CH cell + 0.9% NaCl group ($p < 0.01$). There was no significant difference between those in the Normal cell + 0.9% NaCl group and the CH cell + 10 mM DHF group ($p > 0.05$) (Fig. 2A). And after HT22 was exposed to DHF for 24 h, there was no significant difference between those in CH cell + 10 mM DHF group, CH cell + 5 mM DHF group, and Normal cell + 0.9% NaCl group, and that in CH cell + 0.9% NaCl group was significantly higher than other group ($p < 0.01$) (Fig. 2B). After NSC-34 was exposed to DHF for 12 h, the apoptosis rate in CH cell + 10 mM DHF group was significantly lower than that in CH cell + 5 mM DHF group ($p < 0.05$), that in CH cell + 10 mM DHF group was significantly lower than that in CH cell + 0.9% NaCl group ($p < 0.01$). There was no significant difference between those in the Normal cell + 0.9% NaCl group and the CH cell + 10 mM DHF group ($p > 0.05$) (Fig. 2C). After NSC-34 was exposed to DHF for 24 h, there was no significant difference between those in CH cell + 10 mM DHF group, CH cell + 5 mM DHF group, and Normal cell + 0.9% NaCl group ($p > 0.05$), and that in CH cell + 0.9% NaCl group was significantly higher than other group ($p < 0.01$) (Fig. 2D).

After HT22 was exposed to 5 mM DHF for 12 h, the apoptosis rate was significantly higher than that exposed to 5 mM DHF for 24 h, 10 mM DHF for 12 h, and 10 mM DHF for 24 h ($p < 0.05$), and there was no significant difference between the rest of the three conditions. Therefore, 5 mM DHF for 24 h was selected as the concentration and time for subsequent experiments (Fig. 2E). After NSC-34 was exposed to 5 mM DHF for 12 h, the apoptosis rate was significantly higher than that exposed to 5 mM DHF for 24 h, 10 mM DHF for 12 h, and 10 mM DHF for 24 h ($p < 0.05$), and there was no significant difference between the rest of the three conditions. Therefore, 5 mM DHF for 24 h was selected as the concentration and time for subsequent experiments (Fig. 2F). HT22 and NSC-34 cells showed a consistent trend in the results.

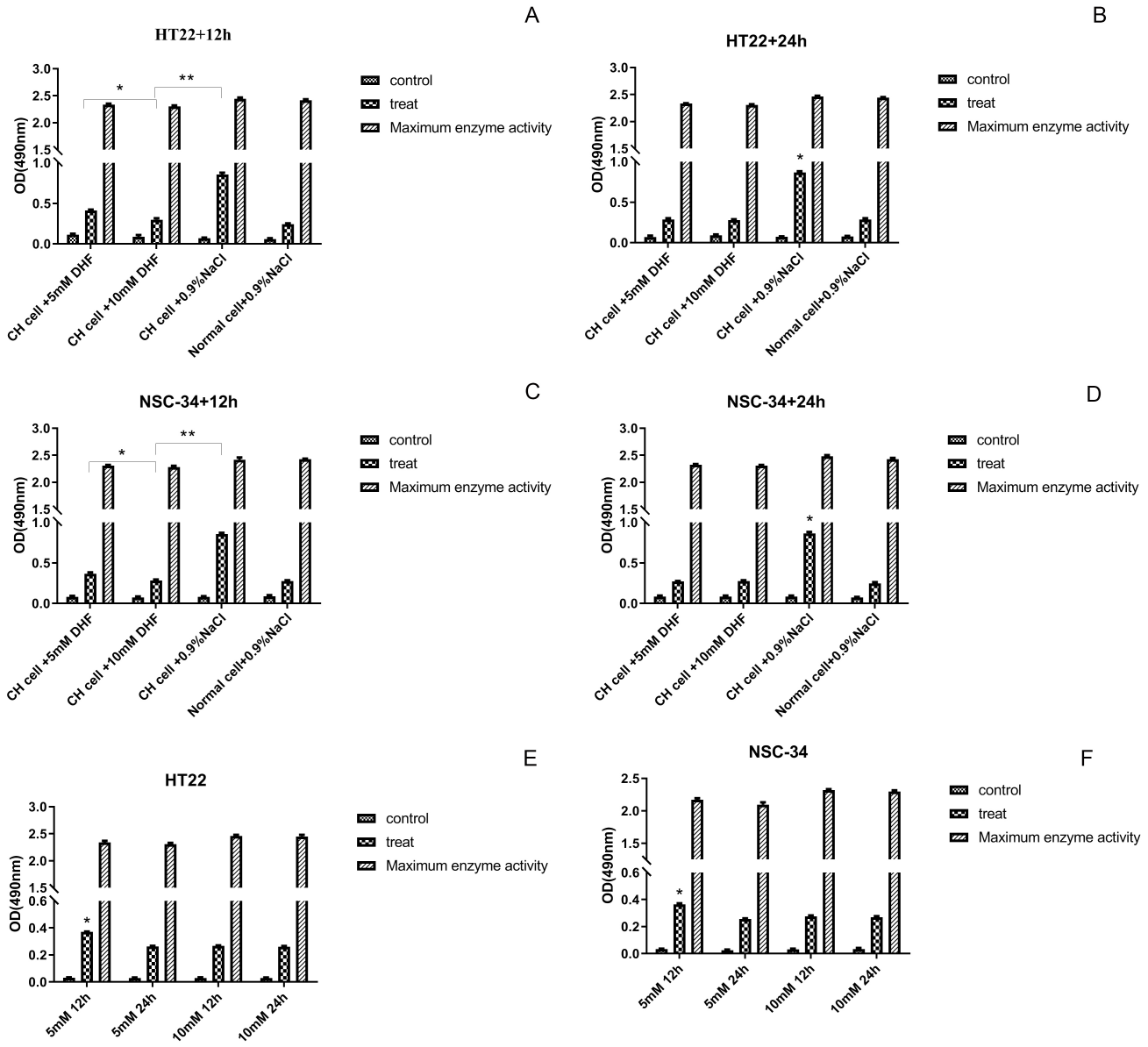

Fig. 2. DHF inhibits the apoptosis of neuronal cells *in vitro*. (A) Injured HT22 exposed to DHF for 12 h. (B) Injured HT22 exposed to DHF for 24 h. (C) Injured NSC-34 exposed to DHF for 12 h. (D) Injured NSC-34 exposed to DHF for 24 h. (E) Injured HT22 exposed to 5 mM DHF and 10 mM DHF for 12 h and 24 h. (F) Injured NSC-34 exposed to 5 mM DHF and 10 mM DHF for 12 h and 24 h, respectively. * represents significant differences between the two groups ($p < 0.05$, n = 3), and ** represents quite significant differences between the two groups ($p < 0.01$, n = 3).

DHF Promotes the Expression of Cellular Neurotrophic Factor-Related Genes and Proteins in Vitro

mRNA expression of BDNF (Fig. 3A), TrkB (Fig. 3B), PI3K (Fig. 3C), AKT (Fig. 3D), MAPK (Fig. 3E) and Erk (Fig. 3F) genes in HT22 and NSC-34 were detected, respectively. The protein expression levels of TrkB and p-TrKB genes in injured HT22 (Fig. 3 G,I) and NSC-34 (Fig. 3H,I) were detected by WB. The results showed that the relative gene expression of BDNF, TrkB, PI3K, AKT, MAPK, and Erk in injured HT22 and NSC-34 in the CH cell + DHF group was significantly higher than that in the CH cell + 0.9% NaCl group ($p < 0.05$). There was no significant difference between the CH cell + DHF group and the Normal cell + 0.9% NaCl group ($p > 0.05$).

The results of WB on TrkB and p-TrKB protein expression levels among all groups showed that there was no significant difference in the TrkB expression level of HT22 and NSC-34 among all groups ($p > 0.05$) while p-TrKB protein expression level of CH cell + DHF group was significantly higher than that of CH cell + 0.9% NaCl group ($p < 0.05$). There was no significant difference between the CH cell + DHF group and the Normal cell + 0.9% NaCl group ($p > 0.05$). As an analog of BDNF, DHF can induce the

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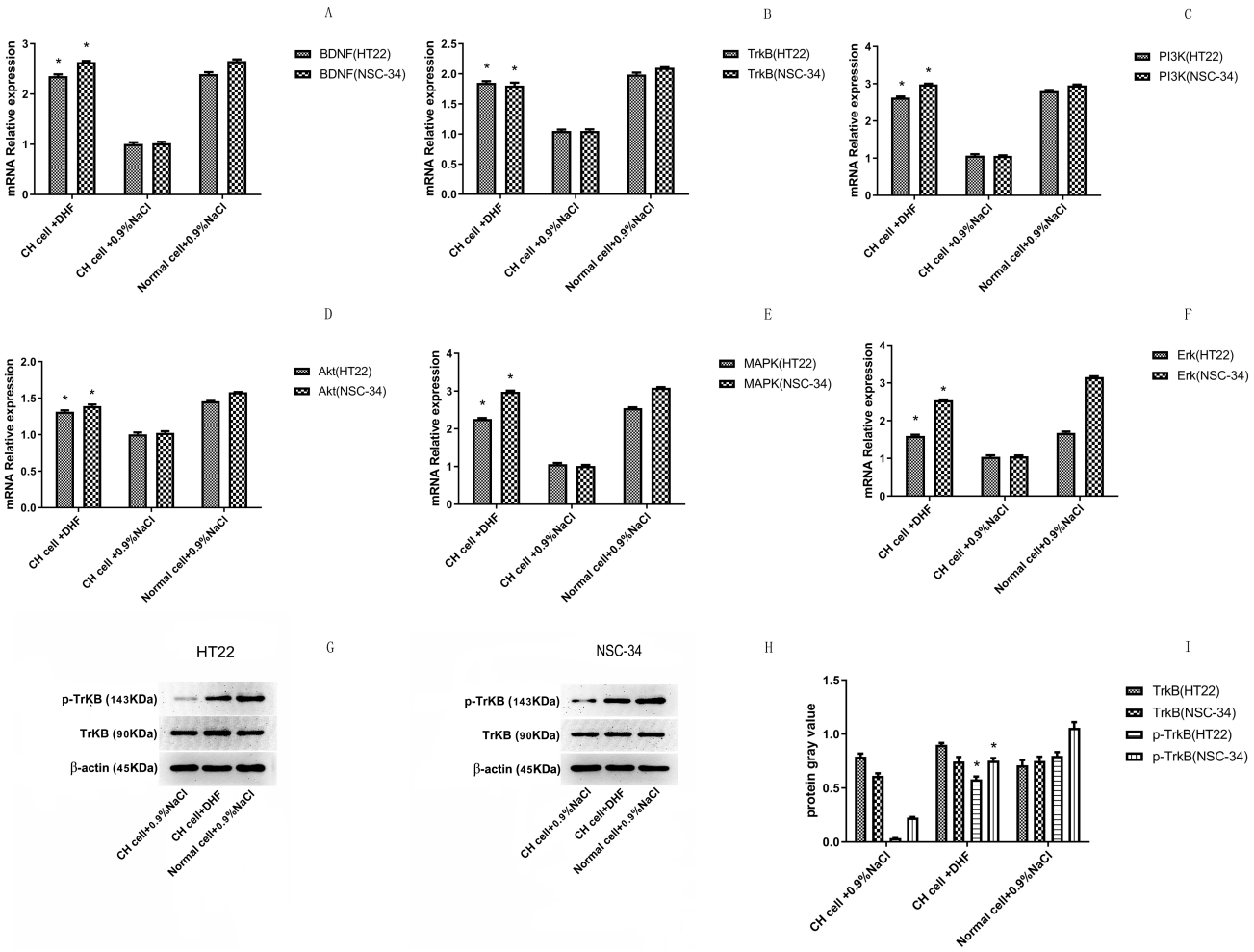

Fig. 3. DHF promotes the expression of cellular neurotrophic factor-related genes and proteins *in vitro*. (A) BDNF expression level in each group of injured HT22 and NSC-34. (B) TrkB expression level in each group of injured HT22 and NSC-34. (C) PI3K expression level of injured HT22 and NSC-34. (D) AKT expression level in each group of injured HT22 and NSC-34. (E) MAPK expression level in each group of injured HT22 and NSC-34. (F) Erk expression level in each group of injured HT22 and NSC-34. (G) Expression level of each histone protein (TrkB, p-TrKB) in injured HT22 cells. (H) Expression level of each histone protein (TrkB and p-TrKB) in injured NSC-34. (I) Grayscale of the expression of each histone protein (TrkB and p-TrKB) in injured HT22 and NSC-34. * represents significant differences between the two groups ($p < 0.05$, n = 3).

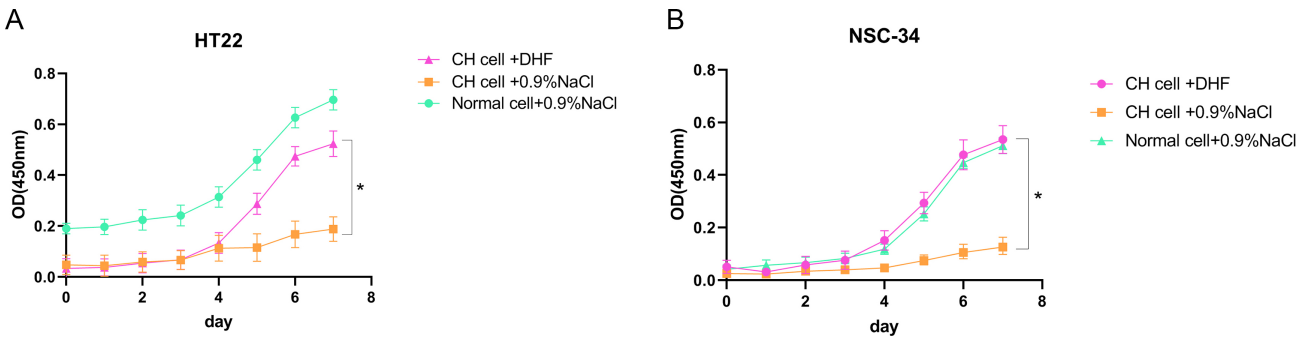

Fig. 4. DHF promotes the proliferative capacity of neuronal cells *in vitro*. (A) Growth curve of HT22 injured treated by DHF, 0.9% NaCl and normal HT22 cells treated by 0.9% NaCl. (B) Growth curve of NSC-34 injured treated by DHF, 0.9% NaCl and normal NSC-34 cells treated by 0.9% NaCl (n = 3). * represents significant differences between the two groups ($p < 0.05$).

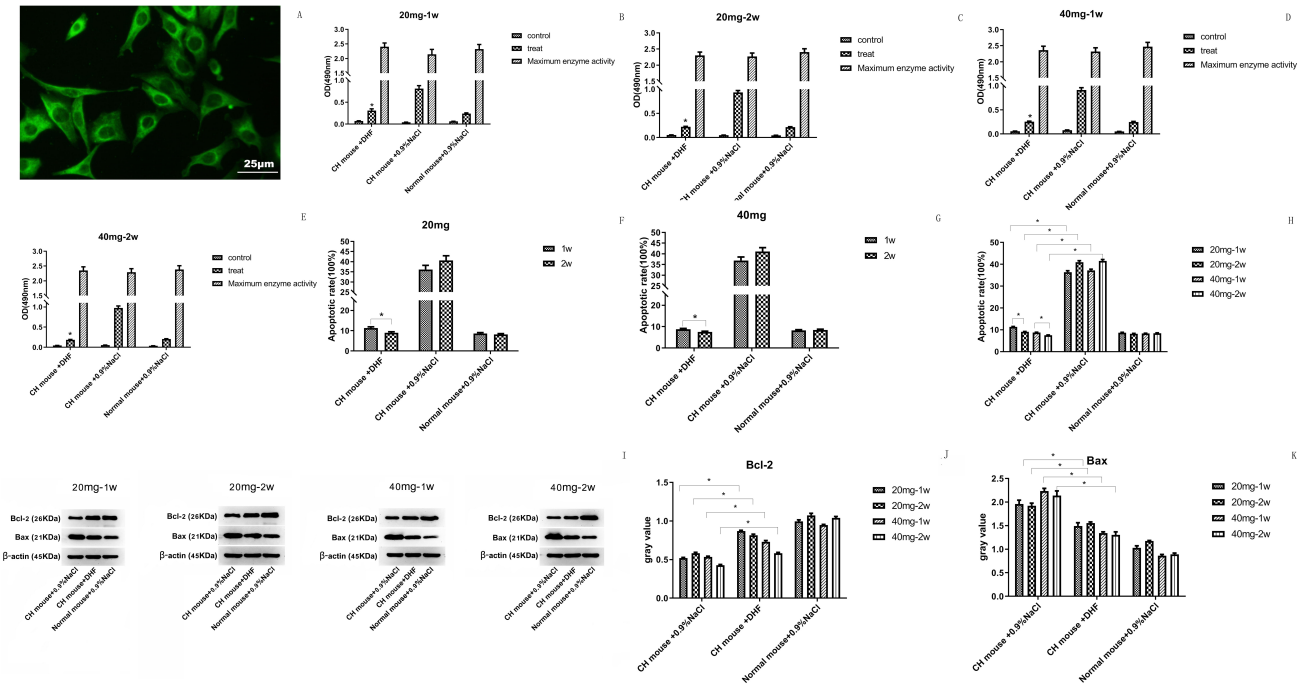

Fig. 5. DHF inhibits the apoptosis of neuronal and glial cells of mice. (A) The neuronal and glial cells of mice imaged based on anti-beta III Tubulin monoclonal and secondary antibody staining. (B) Effect of 20 mg DHF on apoptosis of primary neuronal and glial cells of mice after brain injury for 1 week. (C) Effect of 20 mg DHF on apoptosis of primary neuronal and glial cells of mice after brain injury for 2 week. (D) Effect of 40 mg DHF on apoptosis of primary neuronal and glial cells of mice after brain injury for 1 week. (E) Effect of 40 mg DHF on apoptosis of primary neuronal and glial cells of mice after brain injury for 1 week. (F) Comparison of the apoptosis of primary neuronal and glial cells of mice after 1 and 2 weeks of brain injury treated with 20 mg DHF. (G) Comparison of the apoptosis of primary neuronal and glial cells of mice after 1 and 2 weeks of brain injury treated with 40 mg DHF. (H) Effect of DHF on the apoptosis of primary neuronal and glial cells after 20 mg-1w, 20 mg-2w, 40 mg-1w and 40 mg-2w in mice with brain injury. (I) Results of WB on Bcl-2 and Bax in primary neuronal and glial cells of brain injured mice after intervention with DHF at 20 mg-1w, 20 mg-2w, 40 mg-1w and 40 mg-2w. (J) Analysis on Bcl-2 expression in primary neuronal and glial cells of brain injured mice after intervention with DHF at 20 mg-1w, 20 mg-2w, 40 mg-1w and 40 mg-2w. (K) Analysis on Bax expression in primary neuronal and glial cells of brain injured mice after intervention with DHF at 20 mg-1w, 20 mg-2w, 40 mg-1w and 40 mg-2w. * represents significant differences between the two groups ($p < 0.05$, n = 10).

dimerization of TrkB and activate its downstream signaling molecules, including AKT, thus promoting the expression of TrkB and AKT and inhibiting the apoptosis of neuronal cells [12].

DHF Promotes the Proliferative Capacity of Neuronal Cells in Vitro

The CCK-8 assay was used to detect the cell proliferation. In terms of the proliferation trend, the proliferation curve of the CH cell + DHF group was close to normal cells, that in the CH cell + 0.9% NaCl cells was slow ($p < 0.05$), and those of HT22 and NSC-34 were consistent. See Fig. 4.

DHF Inhibits the Apoptosis of Neuronal and Glial Cells of Mice

A C57BL/6J mouse ICH model was established by intraperitoneal injection of 20–40 mg/kg DHF or the equivalent amount of normal saline daily for one to two weeks. After the last injection, the mice were euthanized 24 h later, the brains were removed, and the tissues were cut up. After digestion with 0.25% pancreatin, the number of 1×10^5 neuronal and glial cells were extracted and imaged based on anti-beta III Tubulin monoclonal and secondary antibody staining (Fig. 5A), and the apoptosis of neuronal and glial cells was detected by LDH kit. DHF intervention agent was added to the model groups at 20 mg-1w (week) (Fig. 5B), 20 mg-2w (Fig. 5C), 40 mg-1w (Fig. 5D) and 40 mg-2w (Fig. 5E), respectively, to compare the difference between the experimental groups and the control group. The results showed that the CH mouse + DHF group was significantly lower than the CH mouse + 0.9% NaCl group ($p < 0.05$). With increasing doses of DHF and longer time, the apoptosis rate decreased. Compared with the apoptosis rate after treating with 20 mg DHF for 1 week, that after treating with 20 mg DHF for 2 weeks was significantly lower ($p < 0.05$, Fig. 5F); compared with the apoptosis rate after treat-

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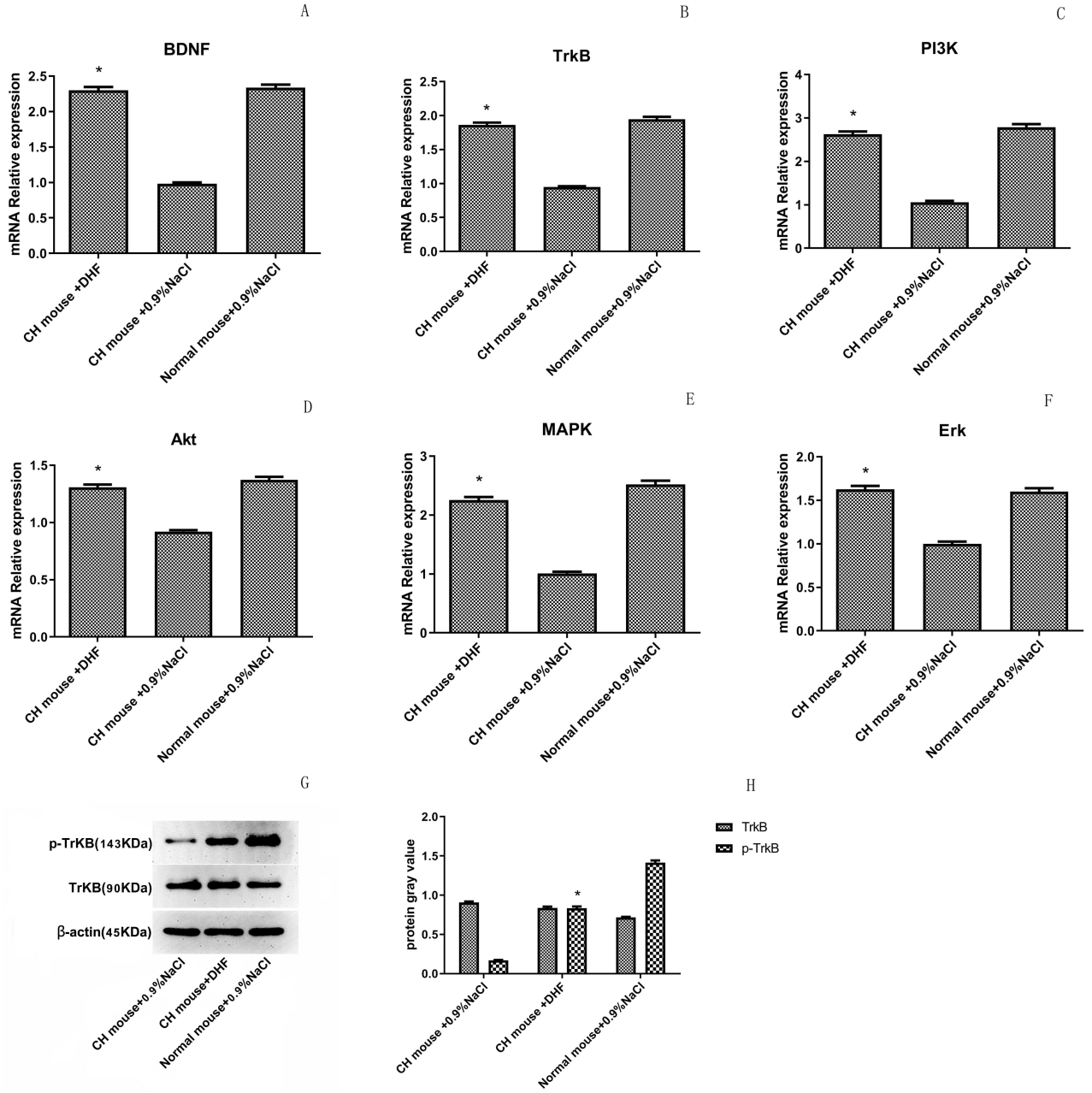

Fig. 6. DHF promotes the expression of cellular neurotrophic factor-related genes and proteins *in vivo*. (A) Expression level of BDNF in primary neuronal and glial cells of mice with ICH after DHF intervention. (B) Expression level of TrkB in primary neuronal and glial cells of mice with ICH after DHF intervention. (C) Expression level of BP13K in primary neuronal and glial cells of mice with ICH after DHF intervention. (D) Expression level of AKT in primary neuronal and glial cells of mice with ICH after DHF intervention. (E) Expression level of MAPK in primary neuronal and glial cells of mice with ICH after DHF intervention. (F) Expression level of Erk in primary neuronal and glial cells of mice with ICH after DHF intervention. (G) Results of WB on TrkB and p-TrkB proteins in primary neuronal and glial cells of mice with ICH after DHF intervention. (H) Analysis on the expression level of TrkB and p-TrkB proteins in primary neuronal and glial cells of mice with ICH after DHF intervention. * represents significant differences between the two groups ($p < 0.05$, n = 10).

ing with 40 mg DHF for 1 week, that after treating with 40 mg DHF for 2 weeks was significantly lower ($p < 0.05$, Fig. 5G,). There was no significant difference between the apoptosis rate of 20 mg DHF for 2 weeks and 40 mg DHF for 2 weeks ($p > 0.05$, Fig. 5H).

Therefore, 40 mg/kg was selected as the therapeutic dose for the follow-up experiments, with a treatment time of 2 weeks. The results of WB and statistical analysis on apoptosis-related genes, Bcl-2 and Bax, showed that the expression of Bcl-2 protein in CH mouse + DHF group was

significantly higher than that in CH mouse + 0.9% NaCl group ($p < 0.05$, Fig. 5I,J), the expression of Bax protein in CH mouse + DHF group was significantly lower than that in CH mouse + 0.9% NaCl group ($p < 0.05$, Fig. 5I,K). Compared with the Normal mouse + 0.9% NaCl group, there were no significant differences in the expression of Bcl-2 and Bax proteins in the CH mouse + DHF group ($p > 0.05$, Fig. 5I,J). The gray level of Bcl-2 and Bax showed no significant differences in the CH mouse + DHF group of 20 mg-1w, 20 mg-2w, 40 mg-1w, 40 mg-2w.

DHF Promotes the Expression of Cellular Neurotrophic Factor-Related Genes and Proteins in Vivo

The mRNA expression of BDNF (Fig. 6A), TrkB (Fig. 6B), PI3K (Fig. 6C), AKT (Fig. 6D), MAPK (Fig. 6E) and Erk (Fig. 6F) genes was detected by qPCR. The protein expression level of TrkB and p-TrKB genes was determined by WB (Fig. 6G,H). The results showed that RNA expression of BDNF, TrkB, PI3K, AKT, MAPK, and Erk was significantly higher in the CH mouse + DHF group than that in the CH mouse + 0.9% NaCl group ($p < 0.05$), without a significant difference between CH mouse + DHF group and Normal mouse + 0.9% NaCl group ($p > 0.05$). The results of WB on expression levels of TrkB and p-TrkB proteins in each group showed no significant difference in the expression level of TrkB in each group ($p > 0.05$). The expression level of P-TrKB in the CH mouse + DHF group was significantly higher than that in the CH mouse + 0.9% NaCl group ($p < 0.05$). There was no significant difference between the CH mouse + DHF group and the Normal mouse + 0.9% NaCl group ($p > 0.05$).

Discussion

Non-traumatic ICH is caused by cerebral parenchymal hemorrhage for arterial vascular rupture, most commonly (>80%) due to the influence of cerebral small vessel disease (SVD) on small arterioles [13]. Deep perforated artery diseases (also known as hypertensive arteriopathy or arteriolosclerosis) and cerebral amyloid angiopathy (CAA) are the most common forms of ICH caused by sporadic SVD [14]. Brain edema after ICH can be divided into periematoma edema (PHE) and intratoma edema. After the initial injury of small bleeding, most patients can survive, while secondary injury can lead to severe neurological deficits and even death [15]. The treatment of nerve injury after intracerebral hemorrhage remains to be explored.

Neurotrophic factors are potential molecules that can inhibit or even reverse neurodegeneration [16]. BDNF activates the TrkB membrane receptor, which regulates neuronal development, synaptic plasticity, prevention of oxidative stress, and apoptosis [17]. BDNF is a protein synthesized mainly in neurons and is widely distributed in the central nervous system [18]. It plays a key role in neuronal survival, differentiation, growth, and development. Increasing evidence suggests that BDNF exerts its physiologically beneficial effects mainly by binding to its specific receptor, TrkB [19]. Previous research has shown that BDNF/TrkB ligand-receptor reaction induces the receptor's dimerization, activating the receptor's tyrosine kinase region. Tyrosine (Y) in this region is automatically phosphorylated, as is Y484 and Y785 in the surrounding region, triggering the receptor to bind to protein zygotes and PLC proteins in the SHC PTB region, and the signal is transmitted downward. Downstream signaling pathways include the Ras/MAPK pathway, PI3K/3-Phosphoinositide-dependent protein kinase kin-1 (PDK1)/AKT pathway, and PLC pathway 3, associated with increased release and activation of Ca^{2+} [20]. Preclinical evidence strongly supports that BDNF may be useful as a therapeutic agent for several neurological disorders. However, the results of several clinical trials using recombinant BDNF have been disappointing. This could be due to poor delivery of BDNF, a short half-life *in vivo*, and an inability to cross the blood-brain barrier [21].

7,8-DHF is a kind of natural flavonoid, as an orally bioavailable BDNF mimic, which can bind TrkB receptor of BDNF with high affinity and specificity, activate its downstream signaling cascade, cause the activation of downstream PI3K/AKT, MAPK or Erk pathways, increase the expression of PI3K, MAPK and AKT, and resist the apoptosis of injured neuronal and glial cells, playing a certain role in neuroprotection [22]. 7,8-DHF can promote neurogenesis in the dentate gyrus, increase the density of the dendritic spine, and play neurotrophic roles in various developmental disorders by penetrating the blood-brain barrier. Study has shown that 7,8-DHF has been used for schizophrenia, where DHF-7 increases the expression and phosphorylation of brain-derived neurotrophic factors to activate TrkB/Fyn/NMDAR2B and Raf/MEK/ERK signaling pathways [23]. It has been shown that in retinal cells, treatment with 7,8-DHF prevents excitotoxicity and oxidative stress, ameliorates high glucose-induced apoptosis in diabetic cells, and protects the immature retina from hypoxic-ischemic injury through Müller glial regeneration and MAPK/ERK activation. Furthermore, it has been shown that *in vivo*, 7,8-DHF improves spatial learning and memory in older rats with cognitive impairment and prevents synaptic loss and memory deficits in a mouse model of Alzheimer's disease [24]. 7,8-DHF has been widely studied in recent years, mainly for the treatment of diseases related to the central nervous system (CNS), such as Alzheimer's disease, cognitive dysfunction, depression, and Parkinson's disease [18]. Lower levels of BDNF-TrkB signaling were frequently reported in depressed subjects. Up-regulating BDNF-TrkB by pharmacological agents in the prefrontal cortex (PFC) and hippocampus allows people to remain resilient to inescapable stress and alleviate depressive symptoms [25,26]. In this study, it was observed by establishing a model of neuronal cell injury *in*

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vitro and *in vivo* that 7,8-DHF can activate or phosphorylate the BDNF receptor, TrkB, with specific manifestations including increased expression of RNA such as TrkB and P-TrkB, which leads to the activation of downstream PI3K/AKT, MAPK or Erk pathways, and increased expression of genes, such as PI3K, MAPK and AKT, which can resist the apoptosis of injured neuronal and glial cells and promote the proliferation of neuronal and glial cells, playing a specific neuroprotective role.

As found in the present study using a model of injured neuronal cells caused by Hemin, the apoptosis was significantly reduced by treatment with 7,8-DHF of injured neuronal cells. After treatment with 5 mM DHF and 10 mM DHF for 12 h and 24 h, the data of apoptosis rate at 5 mM DHF for 12 h, 5 mM DHF for 24 h, 10 mM DHF for 12 h and 10 mM DHF for 24 h showed a decreasing trend with the increase of DHF concentrations and time, showing that the effects of 7,8-DHF were dose-dependent and protected cells against the occurrence of apoptosis. It was also observed that 7,8-DHF can promote the expression of p-TrkB and downstream genes related to the activation of PI3K/AKT, MAPK or Erk pathways (BDNF, TrkB, PI3K, AKT, MAPK, and Erk), which has been confirmed in the animal model trials. In an animal ICH model, the expression of p-TrKB was 2.8-fold higher after DHF intervention, and from this, it was inferred that 7,8-DHF promoted the phosphorylation of TrkB. Meanwhile, the expression of genes related to the activation of downstream PI3K/AKT, MAPK, or ERK pathways (BDNF, TrkB, PI3K, AKT, MAPK, and ERK) was elevated compared with the control group, which was higher. From this, it was inferred that it is likely that the protective effects of 7,8-DHF on injured neuronal cells are mediated by the activation of downstream signaling pathways, such as PI3K/AKT and MAPK/ERK. After intraperitoneal injection of 7,8-DHF in mice of an animal ICH model, the apoptosis of brain neuronal and glial cells in mice was decreased, and the expression of Bcl-2, a suppressor gene, significantly increased. In contrast, the expression of Bax, a pro-apoptotic gene, significantly decreased. In addition, in this study, it was observed that treatment with 7,8-DHF increased the proliferation capacity of neuronal cells (HT22 and NSC-34), and the proliferation curve of injured neuronal cells after DHF treatment was close to that of normal cells.

In similar studies by other investigators, Tsai T *et al*. [9] performed studies in which mouse embryonic motoneurons were cultured for 5 days in the presence of three different doses of 7,8-DHF (400 pM, 40 pM and 4 pM), BDNF (40 pM, positive control), and no stimulator. The percentage of apoptotic cells significantly decreased when motoneurons were cultured at 40 pM 7,8-DHF compared with controls (27.7 ± 4.0%) [9], which were in the same trend as the results of the present study. Tsai T *et al*. [9] investigated the activation of PI3K/AKT and MAPK pathways in motor neurons treated with 7,8-DHF. Unlike the present study, the analysis of both pathways demonstrated that the PI3K/AKT pathway was activated in cultured motor neurons, which was not the MAPK pathway [11], with a result contrary to the present study.

A previous report by Wu CH *et al*. [27] showed that treatment with 7,8-DHF can significantly improve the apoptosis of brain neurons in mice with ICH after 3 days of treatment (17.7 ± 0.6% vs 21.3 ± 1.0%). Wu CH *et al*. [27] assessed the changes in the apoptosis of brain tissue protection induced by 7,8-DHF. Compared with the control group, 7,8-DHF significantly reduced the number of TUNEL-positive cells (56.8 ± 3.1%, 68.9 ± 2.5%). At the same time, the ratio between the anti-apoptotic and pro-apoptotic Bcl-2 family members (Bcl-2 and Bax) was also measured because the imbalance of anti-apoptotic and pro-apoptotic Bcl-2 family proteins is an important cause of apoptosis. Compared with the control group, DHF 20 significantly increased the Bcl-2/Bax ratio (47% vs 325%) [27]. The above results are consistent with the results of the present study. The results from Wu CH *et al.* [27] suggest that 7,8-DHF induced the activation of TrkB and downstream PI3K/AKT signaling pathway, while it did not affect the Erk signaling pathway. 7,8-DHF significantly increased TrkB phosphorylation to 151% of its carrier levels on Day 4 of treatment, and the levels of the two AKT phosphorylation forms in the 7,8-DHF group were significantly higher than those in the control group on Day 4 (phospho-AKT Ser473: 198% of carrier levels; phospho-AKT Thr308: 220% of the carrier level). In contrast to our study, they did not detect any difference in Erk1/2 protein level after treatment with 7,8-DHF at all test time points [27].

Increasing evidence demonstrates a critical role of TrkB signaling in promoting neuronal survival [27]. Activation of TrkB prevents neuronal degeneration in various *in vitro* neuronal injury models [28]. Activation of TrkB also reduces functional and histologic damage after experimental cerebral ischemia [29,30]. Although the activation of TrkB signaling by small molecule BDNF mimics can reverse the motor deficits induced by neuronal injury in rats, it fails to alleviate brain tissue injury, and the exact mechanism of its protective effect remains unclear. Our study found that TrkB expression was reduced in mice after ICH neuronal injury, and intravenous injection of 7,8-DHF can activate TrkB, downstream PI3K/AKT, and other pathways, thus protecting injured neurons.7,8-DHF is a BDNF substitute substance that can compensate for the deficiency of BDNF in treating nerve injury and is a potential treatment mode for nerve injury [31]. Other non-neurological effects or side effects of 7,8-DHF have not been well studied, thus limiting clinical use. To study the impact of 7,8-DHF on the body in multiple systems is beneficial to the clinical application of 7,8-DHF.

Conclusion

In conclusion, these results show that the activation of 7,8-DHF on TrkB is realized by activating downstream genes related to the PI3K/AKT, MAPK, and Erk pathways. These pathways can resist the apoptosis of injured neuronal cells and promote their proliferation, thus playing a neuroprotective role to a certain extent. 7,8-DHF is a potential treatment method for neuronal injury and can be a substitute for BDNF, with the potential to make up for the deficiency of BDNF in treating neuronal injury.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

(I) Conception and design: HZ, LW, NW; (II) Administrative support: KW, RH; (III) Provision of study materials or patients: HZ, NW; (IV) Collection and assembly of data: KW, RH; (V) Data analysis and interpretation: LW, HZ; (VI) Manuscript writing: all authors; (VII) Final approval of manuscript: all authors. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was approved by the Review Committee and reviewed by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University ((Qi) Ethics Review [2019] No. 1201), and all the patients involved in the project signed the informed consent forms. The study was carried out in accordance with the Helsinki Declaration. All animal experiments were approved by the Ethical Committee of the Second Affiliated Hospital of Qiqihar Medical University ((Qi) Ethics Review [2019] No. 1201).

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Conflict of Interest

The authors declare no conflict of interest.

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