Berberine promotes nerve regeneration through IGFR-mediated JNK-AKT signal pathway

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Abstract. Berberine presents therapeutic ability for various central nervous system disorders, including Alzheimer's disease and cerebral ischemia. The present study investigated the role of berberine in nerve regeneration and analyzed the potential mechanism mediated by berberine in hippocampal pyramidal neurons. Reverse transcription-quantitative poylmerase chain reaction, western blot, TUNEL assay and immunofluorescence were used to analyze the therapeutic effects of berberine on nerve regeneration. Berberine treatment increased growth and viability of hippocampal pyramidal neurons. Berberine treatment inhibited apoptosis of hippocampal pyramidal neurons and increased apoptosis regulator Bcl-2 and Bcl-w expression. Neuroinflammation of tumor necrosis factor α, interleukin (IL)1β, IL6 levels and autophagy-related proteins microtubule-associated proteins 1A/1B light chain 3B, autophagy related 16 like 1 and autophagy related 7 were downregulated by berberine treatment in hippocampal pyramidal neurons. Notably, study has found that berberine increased insulin-like growth factor receptor (IGFR) and decreased c-Jun N-terminal kinase (JNK) and protein kinase B (AKT) expression in hippocampal pyramidal neurons. IGFR antagonist abolished berberine-increased growth of hippocampal pyramidal neurons. In conclusion, these results indicate that berberine can promote nerve regeneration through IGFR-mediated JNK-AKT signal pathway.

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Introduction

Many physiological changes have been observed after peripheral nerve injury including degeneration of nerve cell, dedifferentiation, disintegration of axon, and dedifferentiation of Schwann cell (1). Peripheral nerve injury is a common disease in clinic practice and damage to the nervous system is impacting approximately 20 million people in United States (2). Nerve regeneration plays an important role after peripheral nerve injury in both the central and peripheral nervous systems (3). However, adult mammalian central nervous system axons generally do not regenerate or repair the damaged neuron (4). Therefore, exploring nerve regeneration strategies should invested, including a deep fundamental understanding of the nerve regeneration process and potential mechanism in the processes of natural regeneration of axon.

Berberine is an alkaloid extracted from plants, which presents antibiotic and anti-inflammatory effects (5). Currently, berberine also exerts neuroprotective effects and protects neurons against neurotoxicity by promoting axonal regeneration in the injured nerves of the peripheral nervous system (6). Findings have provided further understanding that berberine action highlighted the therapeutic potential in the treatment of a wide range of neurological disorders by decreasing the 4-AP-induced phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and synapsin I (7). In addition, study has indicated that berberine increased the survival of hippocampal precursor cells and differentiated neurons by promoting neuronal differentiation (8). Furthermore, Study has reported that berberine has therapeutic ability for central nervous system disorders such as Alzheimer's disease and cerebral ischemia. However, the potential mechanism mediated by berberine in nerve regeneration has not been investigated.

In the present study, we investigated the role of berberine in nerve regeneration and analyzed the potential mechanism mediated by berberine in hippocampal pyramidal neurons. We also evaluated the role of berberine on growth, viability and apoptosis of hippocampal pyramidal neurons.

Materials and methods

Animals study. A total of 12 male facial nerve axotomy injury mice model (9) (C57BL/6, 8 weeks old, body weight, 20-25 g) was obtained from the Experimental Animal Center of Jinzhou Medical University. All experiments were conducted under the supervision and with the approval of the Ethic Committee of the Second Hospital of Jilin University (approval no: TSHJLU20140521X). All mice were housed at 23±1˚C, 50±5% humidity with a 12-h light/dark cycle and free access to food and water. All mice were randomly divided into two groups and received treatment with berberine (2 mg/kg/day, Sigma-Aldrich) or the same volume of PBS (Control). The treatments were continued to 20 days. Previous study has found that 5 mg/kg/day of berberine showed protective effect of on doxorubicin-induced acute hepatoraenal toxicity in rats (10). Therefore, we chose 2 mg/kg/day of berberine for the treatment of experimental mice due to the diarrhea caused by high dose of berberine as described previously (11). Berberine did not induce weight gain/loss for the experimental animals during the treatment period (data not shown).

Cells isolation and culture. On day 21, mice were anesthetized using IV Pentobarbital (35 mg/kg) and then sacrificed using cervical decapitation. Hippocampal pyramidal neurons were isolated from berberine- or PBS-treated mice as described previously (12). A total of 3 healthy male C57BL/6 mice (8 weeks old, body weight, 20-25 g) was obtained from the Experimental Animal Center of Jinzhou Medical University and used as control group in cells viability and growth assays cells. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.).

Cells viability assay. Hippocampal pyramidal neurons ($2x10^3$ cells/well) were seeded in 96-well plates and cultured at 37˚C for 12 h. Cells were then treated with 10 μl of MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 3 h at 37˚C. After incubation, purple formazan crystals were dissolved using isopropanol (15 μl, isopropanol). The absorbance was recorded on a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.) at a wavelength of 570 nm. Cells viability was determined by percent of cell viability calculated as the ratio between mean absorbance of three samples and mean absorbance of controls. Cells' morphology was captured using light microscope (Carl Zeiss Microscopy, Göttingen, Germany) at magnification, x400.

Cells growth. Hippocampal pyramidal neurons ($1x10^2$ cells) isolated from berberine- or PBS-treated mice as described above were seeded in 96-well plates and treated with insulin-like growth factor receptor (IGFR)antagonist (IGFRAG; Sigma-Aldrich; Merck KGaA) or PBS 37˚C for 12 h. After incubation, cells were fixed with 10% paraformaldehyde for 30 min at 37˚C and then stained with 2% crystal violet for 30 min at 37˚C. Cells number was cultured at least three random views under a light microscope at magnification, x40.

Western blot analysis. Hippocampal pyramidal neurons ($1x10^7$) were lysed in RIPA buffer (M-PER reagent for the cells and T-PER reagent for the tissues; Thermo Fisher Scientific, Inc.) followed homogenized at 4˚C for 10 min. Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 30 μg protein extracts was electrophoresed on 12% polyacrylamide gradient gels and then transferred to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer (5% milk) prior to incubation with primary antibodies at 4˚C overnight. The primary rabbit anti-rat antibodies used in the immunoblotting assays were: Bcl-2 (1:1,000, cat. no: ab692; Abcam, Cambridge, MA, USA), Bcl-w (1:1,200, cat. no: ab32370; Abcam), JUK (1:1,000, cat. no: ab25901; Abcam), IGFR (1:1,200, cat. no: ab182408; Abcam), AKT (1:500, cat. no: ab151279; Abcam), ATG5 (1:1,000, cat. no: ab108327; Abcam), TNFα (1:1,000, cat. no: ab6671; Abcam), IL1β (1:1,000, cat. no: ab9722; Abcam), IL6 (1:1,000, cat. no: ab7737; Abcam), LC3B (1:1,000, cat. no: ab48394; Abcam), ATG16 L (1:1,000, cat. no: ab187671; Abcam), ATG7 (1:1,000, cat. no: 8558; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (1:2,000, cat. no: ab8226; Abcam). After the incubation, membrane was washed three times in TBST and incubated with HRP-conjugated goat anti-rabbit IgG mAb (PV-6001; ZSGB-Bio, Beijing, China) for 1 h at 37˚C. After three-time washing in TBST, membrane was developed using a chemiluminescence assay system (Roche) and exposed to Kodak exposure films. Densitometric quantification of the immunoblot data was performed by using the software of Quantity-One (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

TUNEL assay. Apoptosis of hippocampal pyramidal neurons were analyzed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (DeadEnd™ Colorimetric Tunel System; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Hippocampal pyramidal neurons ($1x10^5$) were incubated TUNE (DeadEnd™ Colorimetric Tunel System; Promega Corporation). Cells were washed with PBST (Sigma-Aldrich) three times for 5 min at 37˚C followed by incubated with 5% DPAI (Sigma-Aldrich; Merck KGaA) for 15 min at 37˚C. Finally, images were captured with a ZEISS LSM 510 confocal microscope at 488 nm. The apoptosis rate was calculated by using the software of Developer XD 1.2 (Definiens AG, Munich, Germany).

Immunofluorescence staining. Hippocampal pyramidal neurons ($1x10^4$) were fixed with 10% paraformaldehyde for 30 min at 37˚C. Cells were washed with PBST for 10 min at 37˚C and blocked with blocking buffer (0.01 M PBS, 0.1% Triton X-100, and 1% bovine serum albumin) for 30 min at 37˚C. Cells were then incubated with primary antibodies: CNPase (1:11,000, cat no: 5665; Cell Signaling Technology, Inc.) or ATG5 ATG5 (1:1,000, cat no: ab108327; Abcam) in blocking buffer at 4˚C for 12 h. After washing with PBS for 3x10 min, the cells were incubated Alexa Fluor 488/568 FITC goat anti-rabbit secondary antibody (1:1,000, Alexa Fluor® 488; Abcam) for 2 h at 37˚C. After three washes with PBS for 3x10 min. Images of cells were captured on Leica DMI4000B microscope and analysed using the software of Quantity-One (Bio-Rad Laboratories, Inc.).

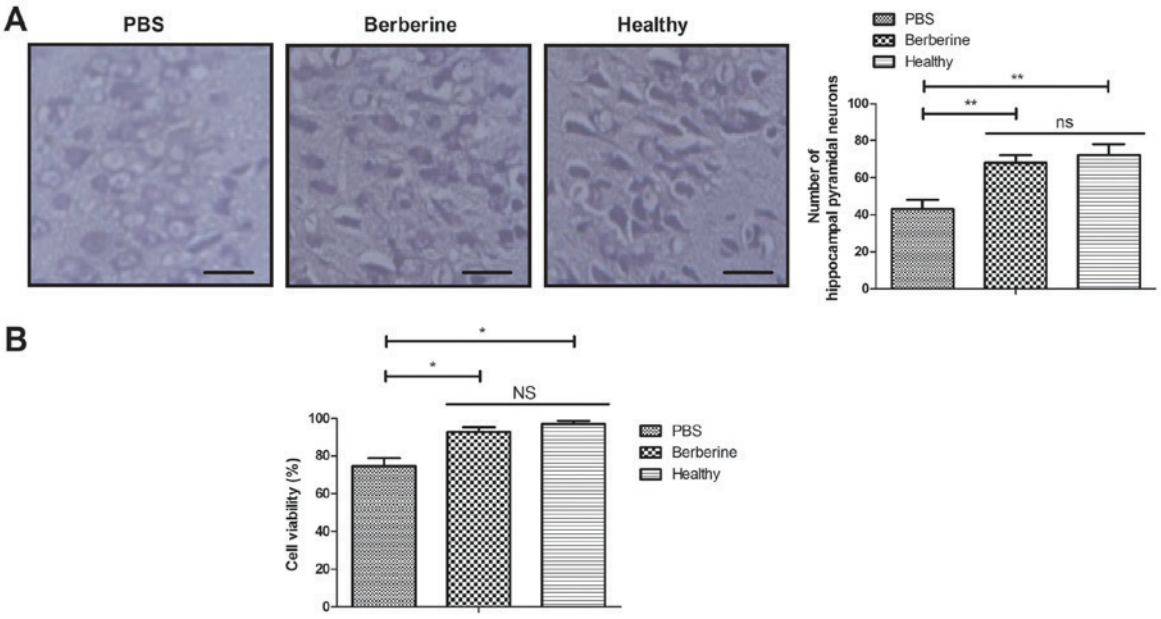

Figure 1. Effects of berberine on growth and viability of hippocampal pyramidal neurons. (A) Hippocampal pyramidal neurons growth was measured by crystal violet staining analysis. Magnification, x400. Scale bar, 100 μm. (B) Viability of hippocampal pyramidal neurons was increased determined by MTT assay. *P<0.05 and **P<0.01 vs. control.

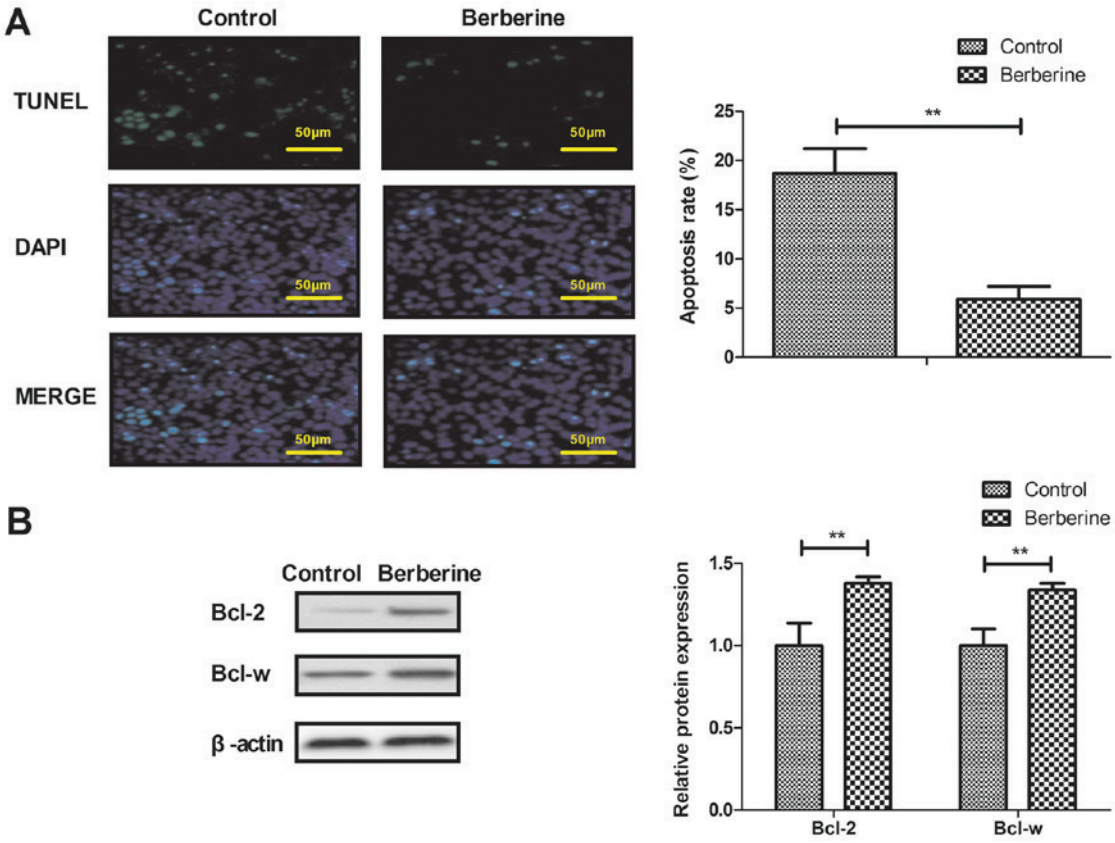

Figure 2. Effects of berberine on apoptosis of hippocampal pyramidal neurons. (A) Apoptosis of hippocampal pyramidal neurons was determined by TUNEL assay. (B) Anti-apoptosis protein Bcl-2 and Bcl-w expression levels in hippocampal pyramidal neurons between berberine and PBs group was determined by western blot. **P<0.01 vs. control.

Statistical analysis. Data are presented as means ± SD of triplicate. All data were analyzed by SPSS v.17.0 software (SPSS, Chicago, IL, USA). Significant differences between two groups were analyzed by two-tail unpaired Student's t-test. Multiple groups differences were analyzed using one-way analysis of variance (ANOVA) followed Tukey HSD test. P<0.05 was considered to indicate a statistically significant difference.

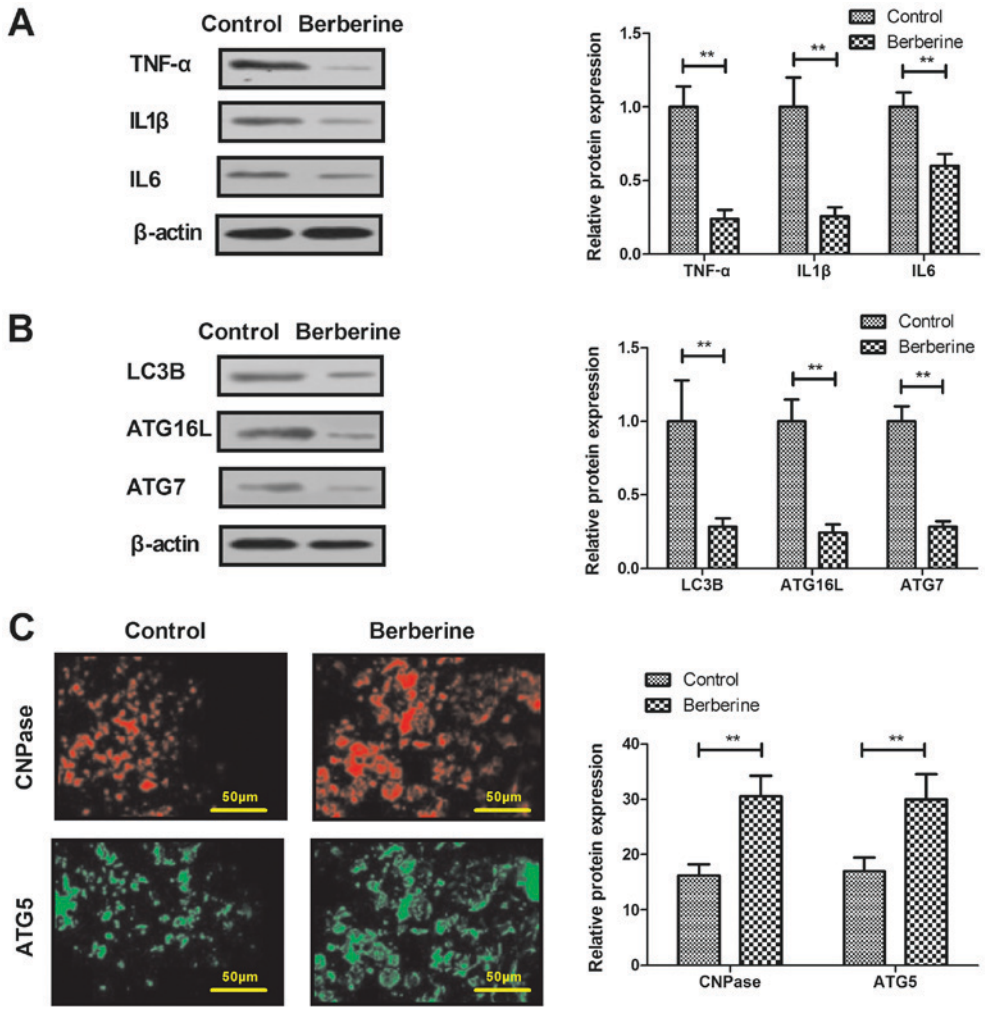

Figure 3. Effects of berberine on neuroinflammation in hippocampal pyramidal neurons. (A) Expression of TNFα, IL1β and IL6 protein level in hippocampal pyramidal neurons between berberine and PBs group was determined by western blot. (B) Expression of LC3B, ATG16L and ATG7 in hippocampal pyramidal neurons between berberine and PBs group was determined by western blot. (C) Expression of CNPase positive oligodendrocyte expressing ATG5 in hippocampal pyramidal neurons between berberine and PBs group was determined by immunofluorescence staining. $^{**}P<0.01$ vs. control.

Results

Berberine increases growth and viability of hippocampal pyramidal neurons. We investigated whether berberine could improve viability of hippocampal pyramidal neurons. Berberine increased growth of hippocampal pyramidal neurons compared to control (Fig. 1A). We found that viability of hippocampal pyramidal neurons was increased determined by MTT assay (Fig. 1B).

Berberine inhibits apoptosis of hippocampal pyramidal neurons. We further analyzed the role of berberine on apoptosis of hippocampal pyramidal neurons. We observed that berberine decreased apoptosis of hippocampal pyramidal neurons (Fig. 2A). Western blot demonstrated that anti-apoptosis protein Bcl-2 and Bcl-w expression levels were increased by berberine in hippocampal pyramidal neurons (Fig. 2B).

Berberine inhibits neuroinflammation in hippocampal pyramidal neurons. The neuroinflammation level was analyzed in hippocampal pyramidal neurons. We observed that berberine decreased TNFα, IL1β and IL6 protein level in hippocampal pyramidal neurons (Fig. 3A). Results demonstrated that berberine decreased autophagy-related proteins LC3B, ATG16L and ATG7 in hippocampal pyramidal neurons (Fig. 3B). We also found that berberine increased CNPase positive oligodendrocyte expressing ATG5 (Fig. 3C).

Berberine promotes nerve regeneration through IGFR-mediated c-Jun N-terminal kinase (JNK) and protein kinase B (AKT) signal pathway. Finally, we explored potential mechanism mediated by berberine for nerve regeneration in hippocampal pyramidal neurons. Results found that berberine increased IGFR and decreased JNK and AKT expression in hippocampal pyramidal neurons (Fig. 4A). IGFR antagonist (IGFRAG) decreased IGFR and increased JNK and AKT expression (Fig. 4B) and abolished berberine-increased growth of hippocampal pyramidal neurons (Fig. 4C).

Discussion

Nerve regeneration plays an important role in the functional recovery after peripheral nerve injury in adulthood (13). Evidences have indicated that berberine possesses

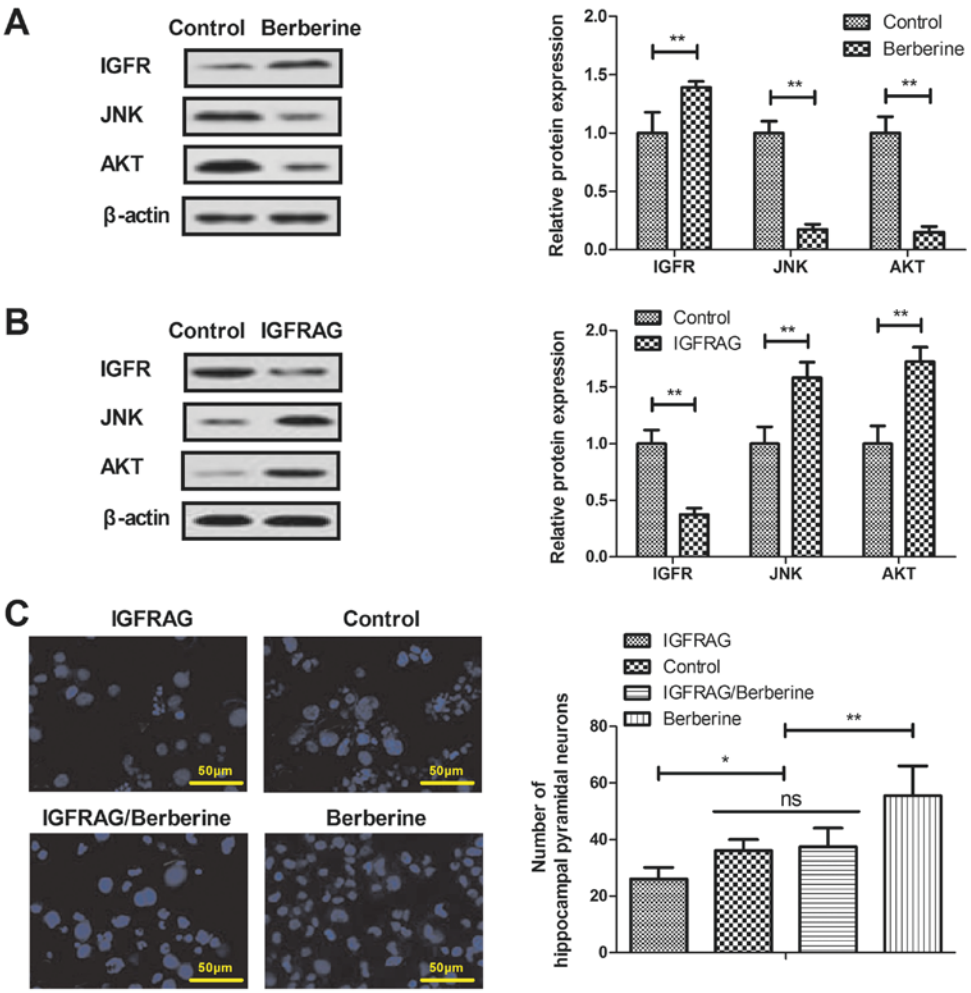

Figure 4. Berberine regulates nerve regeneration through IGFR-mediated JNK-AKT signal pathway. (A) Expression of IGFR, JNK and AKT in hippocampal pyramidal neurons between berberine and PBs group was determined by western blot. (B) Expression of IGFR and increased JNK and AKT in IGFRAG-treated hippocampal pyramidal neurons determined by western blot. $^{**}P<0.01$ vs. control. (C) Growth of hippocampal pyramidal neurons after treatment with IGFRAG measured by crystal violet staining analysis. $^{*}P<0.05$ vs. IGFRAG, $^{**}P<0.01$ vs. control or berberine. IGFR, insulin-like growth factor receptor; IGFRAG, IGFR antagonist.

anti-inflammation and anti-apoptosis function and has protective function in neuronal degeneration and central nervous system injury (14-16). In this study, we showed that berberine increased the viability hippocampal pyramidal neurons and promoted regeneration of hippocampal pyramidal neurons. We reported that berberine treatment decreased the neuroinflammation and autophagy-related protein in hippocampal pyramidal neurons. Notably, results indicate that berberine promotes nerve regeneration through IGFR-mediated JNK-AKT signal pathway.

Currently, repair of peripheral nerve defects is hampered, which is a serious problem and significantly affects patients' life (17). Study has showed that berberine acts as a stimulus of preconditioning that exhibits neuroprotection via promoting autophagy and decreasing anoxia-induced apoptosis (18). Research has observed that berberine prolonged gastrointestinal transit and time to diarrhea in a dose-dependent manner, and significantly reduced visceral pain in the mouse models mimicking diarrhea (19). However, toxicology effects of Berberis, including nausea, emesis, salivation, diarrhea, muscular tremor and paralysis, also have reported in a review (11). Therefore, this study chose 2 mg/kg/day of berberine for the treatment of facial nerve axotomy injury mice, which did not appear diarrhea for all mice. Previous report also found that berberine could reduce traumatic brain injury-induced brain damage by limiting the production of inflammatory mediators by glial cells, rather than by a direct neuroprotective effect (20). In this study, results indicated that berberine inhibited neuroinflammation TNFα, IL1β and IL6 and decreased apoptosis of hippocampal pyramidal neurons. Furthermore, berberine stimulated autophagy and inhibited apoptosis via modulating the autophagy-associated proteins and apoptosis-modulating proteins, which exhibited neuroprotection via promoting autophagy and decreasing anoxia-induced apoptosis (18). Our results showed that berberine promoted autophagy-related proteins LC3B, ATG16L and ATG7 in hippocampal pyramidal neurons. Report has indicated that berberine upregulated CNPase positive oligodendrocyte expressing ATG5, which promoted neuronal survival (21). Findings in this study showed that berberine increased CNPase positive oligodendrocyte expressing ATG5, which might contribute to the growth of hippocampal pyramidal neurons.

Previously, activation of IGFR-PI3K/Akt signaling can induce Schwann cell proliferation and sciatic nerve

regeneration (22). This study demonstrated that berberine decreased IGFR expression in hippocampal pyramidal neurons. Data suggest that JNK is involved in cavernosal apoptosis during the acute phase after partial cavernosal nerve damage (23). Findings indicated that berberine decreased JNK expression and IGFR antagonist abolished berberine-inhibited JNK expression in hippocampal pyramidal neurons. Gong *et al* have suggested that inhibiting the activation of JNK/Akt signaling pathway could protect hippocampal neurons from apoptosis in ischemic brain injury (24). In this study, results showed that IGFR antagonist increased JNK and AKT expression and abolished berberine-increased growth of hippocampal pyramidal neurons, suggesting that berberine may promote neuronal cells growth during facial nerve axotomy injury mice model. However, this study only analyzed the relationships between berberine and IGFR-mediated JNK-AKT signal pathway, which is a limitation of this study. Further investigations should analyze the associations between berberine and IGFR-mediated signal pathways in hippocampal neurons in ischemic brain injury.

In conclusion, the current study showed the neuroprotective effect of berberine on hippocampal pyramidal neurons apoptosis in facial nerve axotomy injury mice model. Berberine attenuated the neuroinflammation factors, TNFα, IL1β and IL6 in hippocampal pyramidal neurons. We first demonstrated that berberine treatment promoted growth of hippocampal pyramidal neurons. Notably, experimental data implied that berberine may promote nerve regeneration through IGFR-mediated JNK-AKT signal pathway, which may be a potential therapeutic agent for nerve injuries therapy and need to be studied for clinical investigations.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZHN and SYJ performed all experiments in the present study. HHQ, LHY, XQL and LZM analyzed the experimental data. XGM and DLH designed all experiments in the present study.

Ethics approval and consent to participate

This study was approved by the Ethic Committee of the Second Hospital of Jilin University (approval number: TSHJLU20140521X).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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