

# DHI increases the proliferation and migration of Schwann cells through PI3K/AKT pathway and the expression of CXCL12 and GDNF to promote facial nerve function repair

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## Research

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# Abstract

## Background

Danhong injection (DHI) is a commonly used drug in the treatment of cardiovascular and cerebrovascular diseases, and its neuroprotective research has been fully confirmed. Schwann cells, as myelin forming cells of peripheral nerve, play an important role in the process of injury and repair. The purpose of this study was to explore the effect of DHI on Schwann cells and its role in facial nerve injury.

## Methods

RSC 96 Schwann cells were treated with different concentrations (0 –2%) of DHI for different time intervals (12 and 36 h). Effect of DHI on cell viability and migration were determined by CCK8 and Transwell assays. The levels of PI3K-Akt signaling related proteins were measured by western blotting analysis, and the effects of DHI on GDNF and CXCL12 using Western Blot, RT-qPCR, and ELISA assays at the cellular and animal levels, respectively. Then LY294002, an inhibitor of PI3K, was used to study the effect of DHI on cell migration and secretion of CXCL12 and GDNF in RSC96 cells by Transwell, Western Blot, RT-qPCR, and ELISA assays. Finally, facial nerve scoring and S-100 immunofluorescence staining were used to study the therapeutic effects of DHI on facial nerve injury.

## Results

Our study found that DHI can promote the proliferation and migration of RSC96 cells, and this effect is related to the activation of PI3K/AKT pathway. LY294002 inhibits the proliferation and migration of RSC96 cells. Besides, DHI can also promote the expression of CXCL12 and GDNF at gene and protein levels, and CXCL12 is, while GDNF is not, PI3K/AKT pathway-dependent. Animal experiments confirmed that DHI could promote CXCL12 and GDNF expression, and promote facial nerve function recovery and myelin regeneration.

## Conclusion

Our *in vitro* and *in vivo* experiments demonstrated that DHI could promote proliferation and migration of Schwann cells through the PI3K/AKT pathway, and increase the expression of CXCL12 and GDNF to promote facial nerve function repair.

## Background

The facial nerve, the seventh cranial nerve in the human body, comes from the brain and crosses the temporal bone, dividing into five branches that control the sensation and movement of the face. The facial nerve is susceptible to the tumor, infection, trauma, surgery, and other injuries because of its long course[1, 2]. Moreover, the recovery of facial nerve function takes a long time, and results are not satisfactory. The residual facial paralysis often affects people's psychological and social activities[3].

Due to the complexity and difficulty of facial nerve repair and huge clinical demand, peripheral nerve regeneration represented by facial nerve transection injury has become the main focus of research. It was found that peripheral nerve transection injury would first trigger the change of the differentiation state of Schwann cells in the damaged area, which was mainly shown as "repair cells" that support the survival of neurons, guide the regenerative axon to its target, and re-wrap the neurons to form myelin[4]. The repair process involving Schwann cells is mainly divided into four parts :(1) Up-regulated expression of neurotrophic factors and proteins that promote axon growth and neuron survival after injury[5, 6]; (2) Recruit a variety of inflammatory factors and immune cells to the damaged area to clear the damaged debris, providing a continuous source of cytokines and promote the vascular formation of distal nerves[7, 8]; (3) Arranged into a special tubular structure to seal myelin, Remak cells (a reparative Schwann cell) and axons, forming a special repair structure conducive to precise connection[9]; (4) Remove useless cell structures for better nerve repair. Since Schwann cells play an important role in the repair of nerve injury, the mechanism of regeneration, remodeling, or migration of Schwann cells will be helpful for the repair of nerve injury[10].

Danhong injection (DHI) is a modern Chinese medicine made by two kinds of drugs, *Salviae Miltiorrhizae* (Dan Shen) and *Flos Carthami* (Hong Hua) with the raw material dose ratio of 3:1 [11]. The main biologically active components of *Salviae Miltiorrhizae* injection include several kinds of water-soluble bioactive substances (Danshensu, protocatechuic aldehyde, rosemary acid, caffeic acid) and fat-soluble compounds (tanshinone A and cryptotanshinone). For the adult patient, 4-8 ml DHI was diluted in 20 ml 50% glucose injection, or 20-40 ml DHI was diluted in 250 ml 0.9 % normal saline and transfused intravenously[12]. It has many pharmacological activities *in vivo*, such as anti-oxidation, suppressing platelet adhesion and aggregation, improving microcirculation, improving hemorheology, regulating blood lipids, etc[13]. The main bioactive components of *Flos Carthami* injection are hydroxyl safflower yellow pigment A (HSYA) and total flavonoids, which have pharmacological effects such as preventing ischemia-reperfusion injury, improving hemodynamics, reducing free radical generation, and increasing nitric oxide generation[14]. Both drugs have the effect of anti-oxidation and improving blood circulation. They are widely used in clinical practice and numerous clinical observations have confirmed that coronary heart disease, heart failure, stroke, cerebral hemorrhage, vertebrobasilar artery ischemic vertigo, and diabetic peripheral neuropathy receiving DHI therapy got favorable outcome [15-18].

Given the role of DHI in the central nervous system and diabetic peripheral neuropathy, we hypothesized that DHI could promote the repair of facial nerve function by affecting the proliferation and migration of Schwann cells. We first studied the effects of DHI on the proliferation and migration of Schwann cells at the cellular level and explored the underlying mechanisms. Then we studied the biological effects of DHI at the animal level. Finally, our experiments showed that DHI could promote the proliferation and migration of Schwann cells to the injured area through PI3K/Akt pathway, and also promote the expression of GDNF to repair the neurons.

## Materials And Methods

## Cell Culture and Reagents

RSC96 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. They were cultured in DMEM medium (Hyclone, USA) at 37°C and 5% CO<sub>2</sub>, supplemented with 10% fetal bovine serum (Gibco, USA). Cell counting kit 8 (CCK8) was purchased from Beyotime (Shanghai, China). Antibody for PI3K, p-PI3K, AKT, p-AKT1 were purchased from Cell Signaling Technology (Beverly, MA, USA), antibodies for GAPDH, CXCL12, and GDNF were purchased from Beyotime (Shanghai, China), Abcam (Cambridge, MA), and Abclonal (Wuhan, China), respectively. Inhibitor LY294002 was from Selleck Chemicals (Shanghai, China). Danhong Injection was purchased from Buchang Pharmaceutical Company (Shandong, China). RIPA lysate, PMSF, BCA kit, and HRP-labeled secondary antibody were purchased from Beyotime (Shanghai, China). TRIzol reagent was purchased from Takara (Invitrogen, Carlsbad, CA, USA).

## Cell Proliferation Assay

RSC96 cells were plated in 96-well plates at the density of 1000 cells/well. After the cells adhered to the wall, the cells were cultured with different concentrations of DHI (0.2%, 0.5%, 1%, 2%) for different times (12 h and 36 h). Then 10  $\mu$ L CCK8 solution was added into the well and the absorbance at 490nm was detected by microplate reader after incubation for 1 hour at 37°C. The OD values obtained from three measurements were recorded and analyzed.

## Cell Migration Assay

The effect of DHI on the migration of RSC96 cells was studied by the Transwell chamber (Corning, USA) with a diameter of 8- $\mu$ m pore size. 250  $\mu$ L serum-free medium containing  $20 \times 10^4$  RSC96 cells was cultured in the upper chamber of Transwell and 500  $\mu$ L of complete medium containing different concentrations of DHI in the lower chamber. The cells were allowed to migrate for 12 hours and then fixed by 4% paraformaldehyde for 15 min, followed by crystal violet staining for 15 minutes. Cotton swabs were used to erase the non-

migrated cells and then five random fields were photographed and counted under a microscope (Olympus, Japan).

## Protein Extraction and Western Blot

The treated cells were washed with PBS three times, then 200  $\mu$ L RIPA lysate containing protease inhibitors PMSF and cocktail was added and incubated on ice for 15 minutes, then lysate was collected and centrifuged at 12000 rpm for 15 minutes. The supernatant was collected and centrifuged for 15 minutes at 12000 rpm. The concentration of the supernatant was determined by BCA kit, and 5X loading buffer was added into the remaining supernatant and boiled at 100 °C for 10 minutes to denature the protein. A protein of 20  $\mu$ g was loaded on polyacrylamide gel for electrophoresis and then transferred to PVDF membranes (Thermo Fisher, USA). After blocking with 5% non-fat milk in TBST, the membranes were incubated with, anti-PI3K, p-PI3K, anti-AKT, anti-p-AKT, anti-CXCL12, anti-GDNF, and anti-GAPDH at 4°C overnight, followed by incubating with HRP-conjugated secondary antibody at room temperature for 1 hour, washed with TBST, added enhanced chemiluminescent substrates (Millipore, Billerica, MA) and luminescent on Image Lab System (Bio-Rad, USA).

## Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Trizol was added to the cells and tissues washed with pre-cold PBS, which were lysed on ice for 10 minutes, and then 1/5 volume of chloroform was added. After blending, the mixture was centrifuged at 4 °C for 15 minutes at 12000 rpm. The supernatant was added with an equal volume of isopropanol, and centrifuged at 4 °C for 15 minutes again. Wash the precipitate twice with 75% alcohol and finally dissolve the precipitate with DEPC water. The reverse transcription kit was used to synthesize cDNA. RT-PCR on-machine operation (ABI7500) was performed according to the instructions of the SYBR Green quantitative PCR kit. The primers were as follows:

CXCL12 Forward GTGACGGTAAGCCAGTCAGC

CXCL12 Reverse TGCACACTTGTCTGTTGTTGC

GDNF Forward GAACCAAGC-CAGTGTATCTCCT

GNDF Reverse ATCGTCTCT-GCCTTTGTCCTC

GAPDH Forward CAGTGCCAGCCTCGTCTCAT

GAPDH Reverse AGGGGCCATCCACAGTCTTC

Reaction conditions were as follows: 5 min at 95°C, followed by 40 cycles of 30 s. at 95°C, 30 s. at 57°C, and 30 s. at 72°C. The expression level of the target gene was calculated by  $2^{-\Delta\Delta CT}$ .

### **Enzyme-linked Immunosorbent Assay (ELISA)**

The ELISA method for the detection of facial nerve tissues in rats was the same as the previous method[19]. In short, the facial nerve tissue was taken out, and then 100  $\mu$ L RIPA lysate was added to every 50mg nerve tissue block to fully grind the tissue until the tissue mass disappeared. The test was then performed according to the ELISA procedure. The detection of supernatant in cell culture was directly based on the instructions.

### **Experimental Animals and Groups**

The animals used in this experiment were Sprague–Dawley rats, weighing between 180 and 200g. The animals lived 12 hours a day and 12 hours a night, with sufficient water and food. All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. A total of 60 adult male Sprague–Dawley rats were used in this experiment. Rats were randomly divided into five groups (12 rats per group): sham group (Sham), facial nerve injury group (FNI), low dose Danhong injection group (L+DHI), moderate dose Danhong injection group (M+DHI) and high dose Danhong injection group (H+DHI). In the low, moderate and high dose DHI groups, the injection concentrations were 0.5, 1, and 2 ml/kg, which were 5, 10, 20 times than the dose for human being of 0.1 ml/kg, respectively.

### **The facial Nerve Injury Model**

**The rat model was established based on our published articles[19]. Briefly, rats were anesthetized by intraperitoneal injection of 1% Pentobarbital Sodium at a dose of 40 mg/kg. After successful anesthesia, the right posterior auricular incision was made, and the main facial nerve and its three branches were dissected and exposed, and then the facial nerve trunk was clamped with a vascular clamp for 50 seconds. Immediately after injury, the rats in the experimental group were intravenously administered with different concentration of DHI for 28 days.**

The facia nerve function was evaluated referring to previous methods[20], which contains two aspects: Vibrissae observation, eye closing, and blinking reflex observation. The specific scoring criteria are as follows:

Absence of eye blinking and closure scored 1; the presence of orbicular muscle contraction, without blinking reflex, scored 2; 50% of eye closure through blinking reflex, scored 3, 75% of closure scored 4. The presence of complete eye closure and blinking reflex scored 5. The absence of movement and posterior position of the vibrissae scored 1; slight shivering and posterior position scored 2; greater shivering and posterior position scored 3 and normal movement with a posterior position scored 4; the symmetrical movement of the vibrissae, with an anterior position, scored 5. The facial nerve function was evaluated on the 1st day, 7th, 14th, and 28th day after the establishment of the model.

### Immunofluorescence Staining

After 28 days of the experiment, the facial nerve tissues were taken, followed by fixing with 4% polymerized paraffin embedding, slicing, dewaxing, antigen repairing, and serum blocking. Then facial nerve tissue sections were incubated with the primary antibody for 4 degrees overnight, and incubated the second antibody for 2 hours at room temperature the next day. After PBS cleaning, DAPI was added to stain the nucleus, and finally observed under the OLYMPUS fluorescence microscope.

### Statistical Analysis

Data were presented as mean  $\pm$  standard deviation and analyzed by Graphpad Prism 8.0 software. All data were obtained by at least three independent experiments. Statistical analysis was conducted by the t-test and one-way analysis of variance (ANOVA);  $p < 0.05$  was considered to statistical significance.

## Results

### DHI Promotes the Proliferation of RSC96 Cells

The effects of DHI on the proliferation of RSC96 cells were examined using a CCK-8 assay. Since DHI is used every 12 hours in the human body, to observe the sustained effect of DHI on RSC96 cells, we

selected 12 hours and 36 hours as observation points. It can be seen from Figure 1 that after 12 hours of DHI treatment, the proliferation ability of RSC96 cells was higher than that of the control group, but the effect of 0.2% and 0.5% DHI had no statistical significance, and the effect of DHI functioning for 36 hours was similar. Therefore, it is likely that 1% and 2% DHI promoted the proliferation of RSC96 cells.

### **DHI Promotes the Migration of RSC96 Cells**

We further used Transwell assay to observe the effect of DHI on the migration of RSC96 cells. We chose 12 hours as the endpoint of migration and observed the number of migrated cells that were stained purple with crystal violet. As shown in Figure 2, different concentrations of DHI significantly promoted migration in a dose-dependent manner, however, there was no significant difference between 1% and 2% concentrations of DHI. Combined with the effect of DHI on cell proliferation and migration, we chose 1% DHI concentration in subsequent experiments.

### **PI3K/Akt Pathway Is Involved in the Effect of DHI on RSC96 Cells**

Previous studies have shown that PI3K/Akt pathway is closely related to the proliferation and migration of cells. To verify whether PI3K/Akt pathway plays a role in the proliferation and migration of RSC96 cells induced by DHI, we detected the key signal proteins, including PI3K, and AKT. We found that the expression of p-PI3K and p-AKT increased significantly after treatment with DHI, while the expression of PI3K, AKT, and GAPDH remained unchanged (Figure 3). From this experiment, it was suggested that the PI3K/AKT pathway is involved in the effect of DHI on RSC96 cells.

### **DHI Increases the Expression of CXCL12 and GDNF in RSC96 Cells**

CXCL12 is a common chemokine and is closely related to the migration of Schwann cells in our previous study, whereas GDNF is a neurotrophic factor closely associated with nerve injury and repair. To detect the effect of DHI on the secretion of these two factors in RSC96 cells, Western blot, RT-PCR, and ELISA experiments were used to detect the expression of CXCL12 and GDNF in RSC96 cells. As shown in Figure 4A, after DHI treatment for 24 hours, the expression of CXCL12 and GDNF protein was promoted by DHI in a concentration-dependent manner. RT-PCR experiments (Figure 4C, 4D) showed that DHI increased the mRNA expression of CXCL12 and GDNF in RSC96 cells, and ELISA experiments (Figure 4E, 4F) showed that the secretion of CXCL12 and GDNF increased in RSC96 cells.

### **The Enhanced Expression of CXCL12 in RSC96 Cells Induced by DHI Depends on PI3K/AKT Pathway**

Western Blot, RT-PCR, and ELISA experiments were used to investigate the relationship between CXCL12 and GDNF expressed by RSC96 cells and the PI3K/AKT pathway. According to Figure 5A, DHI-promoted CXCL12 expression was inhibited by LY294002, a PI3K protein inhibitor, while GDNF expression was not affected. Besides, the mRNA and secretory expression of CXCL12 also decreased after LY294002, while the mRNA and secretory expression of GDNF did not change significantly (Figure 5C, 5D). In a word, these results indicated that DHI induced CXCL12 expression in RSC96 cells through the PI3K/AKT pathway.

## **DHI Promotes the Proliferation and Migration of RSC96 Cells Through PI3K/AKT Pathway**

Previous studies have found that DHI promotes the expression of PI3K/AKT and CXCL12 in RSC96 cells. To find out whether the changes of PI3K/AKT/CXCL12 induced by DHI are related to the proliferation and migration of RSC96 cells, CCK-8 and Transwell experiments were conducted with LY294002 and CXCL12 antibody combined with DHI, respectively. The results showed that the migration of RSC 96 cells was not significantly affected by LY294002 and CXCL12 antibody alone, but was significantly increased after using DHI. This increased effect was weakened after application of LY294002 and CXCL12 antibodies, thus the migration of RSC96 cells was decreased, however, it was still higher than that of the control group (Figure 6A). Similar results were also found in the CCK8 assay of RSC96 cell viability (Figure 6C). The increased cell proliferation viability caused by DHI was weakened after the addition of LY294002 and CXCL12 antibodies, while the application of LY294002 and CXCL12 antibodies alone had no significant effect on cell proliferation. These results demonstrated that DHI promoted the proliferation and migration of RSC96 cells through PI3K/AKT pathway.

## **DHI Improves the Neurological Function Scores of Facial Nerve-injured Rats**

In order to verify the effect of DHI on the repair of facial nerve injury *in vivo*, different concentration of DHI were injected into rats after facial nerve injury, and facial nerve function scores were performed on the 1st, 7th, 14th, and 28th days. Our results showed that the sham-operated rats were always normal and the scores were always 10 points. According to Figure 7A, facial nerve scores improved to varying degrees in all groups except the sham-operated group. There was no statistical difference among these groups on the 7th day (Figure 7B), however, the score gradually increased and showed statistical significance on the 14th day, and the scores were further increased on the 28th day (Figure 7C, 7D). Facial nerve scores increased with increasing doses of DHI, although no statistical difference was shown at high, medium, and low doses. This indicated that DHI can improve the functional recovery of the facial nerve in a dose-dependent manner. In order to observe a more significant therapeutic effect of DHI, we used the data of high-concentration DHI in subsequent results presentation.

## **DHI Increases the Expression of CXCL12 and GDNF in Facial Nerve-injured Rats**

Cell experiments have confirmed that DHI promotes the production of more CXCL12 and GDNF in RSC96 cells. To further verify this result *in vivo*, the ELISA assay was used to detecting the expression of CXCL12 and GDNF on the 1st, 7th, 14th, and 28th days after facial nerve injury. The results (Figure 8A, 8B) showed that the expression of CXCL12 and GDNF in the sham operation group was maintained at a stable level. The expression of CXCL12 in the FNI group increased after the operation, peaked at about 2 weeks, and then returned to the initial level at about 4 weeks. GDNF continued to increase at 1 day after the operation, and remained at a high level at 4 weeks, however, the increased level slowed down later. In the H+DHI group, the expression levels of CXCL12 and GDNF continued to increase during DHI treatment, and the expression levels were significantly higher than those in the FNI group. This indicated that DHI can also promote the production of CXCL12 and GDNF in facial nerve tissue *in vivo*.

## DHI Increases the Expression of S-100 in Injured Facial Nerve

To detect the state of Schwann cells after facial nerve injury, the immunofluorescence staining test was used to stain the nerve tissue 28 days after the operation. According to results (Figure 9), we found that the S-100 expression in the sham group was uniformly around the nerve fiber bundles, while that in the FNI group was significantly reduced and did not wrap around the nerve fibers. In the H+DHI group, the expression of S-100 was significantly up-regulated compared with that in the FNI group, and the expression was more uniform around the nerve fiber bundles. These results suggested that DHI promoted the re-enfolding of Schwann cells after facial nerve injury and functional recovery.

## Discussion

The facial nerve, the seventh pair of cranial nerves in humans, originates intracranially and branches innervate the region of the head and neck, and is a vulnerable nerve in otolaryngology. Facial nerve injury is often caused secondary to the congenital, infectious, idiopathic, iatrogenic, traumatic, neurological, tumor, or systemic etiology. The incidence of facial nerve injury is approximately 20-30 persons/per year/per 100,000 population[21]. Loss of facial nerve structural continuity caused by nerve injury, not only causes salivation, eating difficulties, eye closure incompletely, but also causes corneal turbidity, blindness, and even facial muscle atrophy and linkage, resulting in expression stagnation and facial appearance damage, seriously affecting patient's quality of life and social activities. At present, the clinical treatment of facial nerve injury is not standardized, and further research is needed to improve the treatment effect. Clinical common medications include steroid hormones to relieve acute facial swelling[22], neurotrophic factors to stimulate the regeneration of neurons[23, 24], and incobotulinumtoxin A to relax stiff muscles[25]. In addition, another common approach is the surgical intervention of the facial nerve through nerve repair, replacement, and transplantation[26]. However, in general, patients with better outcomes also tend to have facial asymmetry and functional impairment. In this study, we focused on the Schwann cells in the process of repair facial nerve injury and the therapeutic effect of DHI on facial nerve injury. Our experiment proved that DHI could promote the proliferation and migration of RSC96 cells through PI3K/Akt pathway, increase the expression of CXCL12 and GDNF, and promote the recovery of facial nerve function in rats.

At the beginning of the study, different concentrations of DHI were used to study the effect of DHI on the proliferation and migration of RSC96 cells, and the appropriate DHI concentration for subsequent trials was selected to be 1%. PI3K/AKT pathway is a common signaling pathway associated with cell proliferation and migration[27]. We studied its changes when DHI acts on RSC96 cells, and found that PI3K/AKT is significantly activated, which was consistent with Zhou's findings in atherosclerosis[28] and Feng's study on cerebral ischemia/reperfusion injury [29] in which DHI activates the PI3K/AKT pathway.

GDNF is recognized as a nerve growth factor that promotes the growth of neurons[24], in addition to our previous studies also found that a factor called CXCL12 can promote the migration of Schwann cells[19]. In order to study the effect of DHI on the secretion of these two cytokines in RSC96 cells, we detected the

effect of DHI on the expression of CXCL12 and GDNF. The results showed that DHI could promote the expression of these two factors at both animal and cell levels. To further study the relationship between PI3K/Akt pathway and the secretion of CXCL12 and GDNF, cell proliferation and migration, the inhibitors of PI3K, LY294002, and CXCL12 antibodies were used for experiments. The results showed that CXCL12 expression significantly decreased after PI3K/AKT pathway was inhibited, while GDNF expression was not affected. Also, cell proliferation and migration were significantly reduced after the PI3K/AKT pathway was inhibited. The proliferation and migration of RSC96 cells induced by DHI at the cellular level have been determined, and we further performed immunofluorescence staining in animals to observe the expression of Schwann cells molecular marker S-100 caused by DHI. We found that DHI significantly promoted the S-100 expression around the damaged facial nerve, that is, Schwann cells significantly increased after DHI treatment. Besides, facial nerve scores also demonstrated that the facial nerve function of rats after DHI treatment was significantly improved.

DHI is a widely used Chinese medicine in cardiovascular and cerebrovascular diseases. It has anti-inflammatory, scavenging free radicals, and circulatory promoting effects [17]. Numerous studies have found DHI has protective effects on the central nervous system, such as in ischemia-reperfusion injury model rats DHI can pass to protect the blood-brain barrier and reversal neutrophils infiltrating to play a protective role. For example, DHI protects the blood-brain barrier and reverse neutrophil infiltration in rat models of cerebral ischemia-reperfusion injury[30], and DHI increases the expression of astrocytes Peroxiredoxin 1 in subarachnoid hemorrhage models and reduces the expression of inflammatory factors such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), thereby reducing hematoma volume and brain water content and improving neurological deficits[18].

Our study demonstrated for the first time the therapeutic effect of DHI on facial nerve injury, which provided an experimental basis for the clinical use of DHI in the treatment of facial nerve injury. Few studies have investigated the application of DHI in peripheral nerve injury. In addition to our study, another study found that DHI can alleviate mechanical abnormal pain in diabetic neuropathy rats by inhibiting the activation of ERK1/2, and DHI can increase the BDNF content in the sciatic nerve[31]. In conclusion, as the pattern diagram shown in Figure 10, our experiments showed that DHI could promote cell proliferation and migration through the PI3K/AKT pathway, and increase the expression of CXCL12 and GDNF, ultimately promoting the functional recovery of the facial nerve.

## Conclusion

In conclusion, our *in vitro* and *in vivo* experiments demonstrated that DHI promotes the proliferation and migration of Schwann cells through the PI3K/AKT pathway, and increase the expression of CXCL12 and GDNF to promote facial nerve function repair.

## Declarations

### Compliance with ethical guidelines

All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

### **Consent to publish**

All authors have agreed to make a submission.

### **Availability of data and materials**

All the original data and materials are kept and responsible by the corresponding author.

### **Competing interests**

The authors declare that they have no conflict of interests.

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### **Authors' contributions**

Dekun Gao and Jingchun He conceived the idea and wrote the manuscript. Dekun Gao and Lianhua Sun performed facial nerve injury model, drug administration, Western blot analysis, and immunofluorescence staining experiments. Xiayu Sun performed ELISA and RT-qPCR experiments. Jun Yang and Jingchun He reviewed and edited the final manuscript.

## **Abbreviations**

**DHI:** Danhong injection

**FNI:** facial nerve injury

**CCK-8:** Cell Counting Kit-8

**DMEM:** Dulbecco's modification of Eagle's medium

**SDS:** sodium dodecyl sulphate

**RIPA:** Radio Immunoprecipitation Assay

**PAGE:** polyacrylamide gel electrophoresis

**ELISA:** enzyme-linked immunosorbent assay

**T-TBS:** tween-20

**PVDF:** polyvinylidene fluoride

**PI3K:** phosphatidylinositol 3-kinase

**AKT:** protein kinase B

**CXCL12:** chemokine (C-X-C motif) ligand 12

**GDNF:** Glial-cell line-derived neurotrophic factor

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## Figures

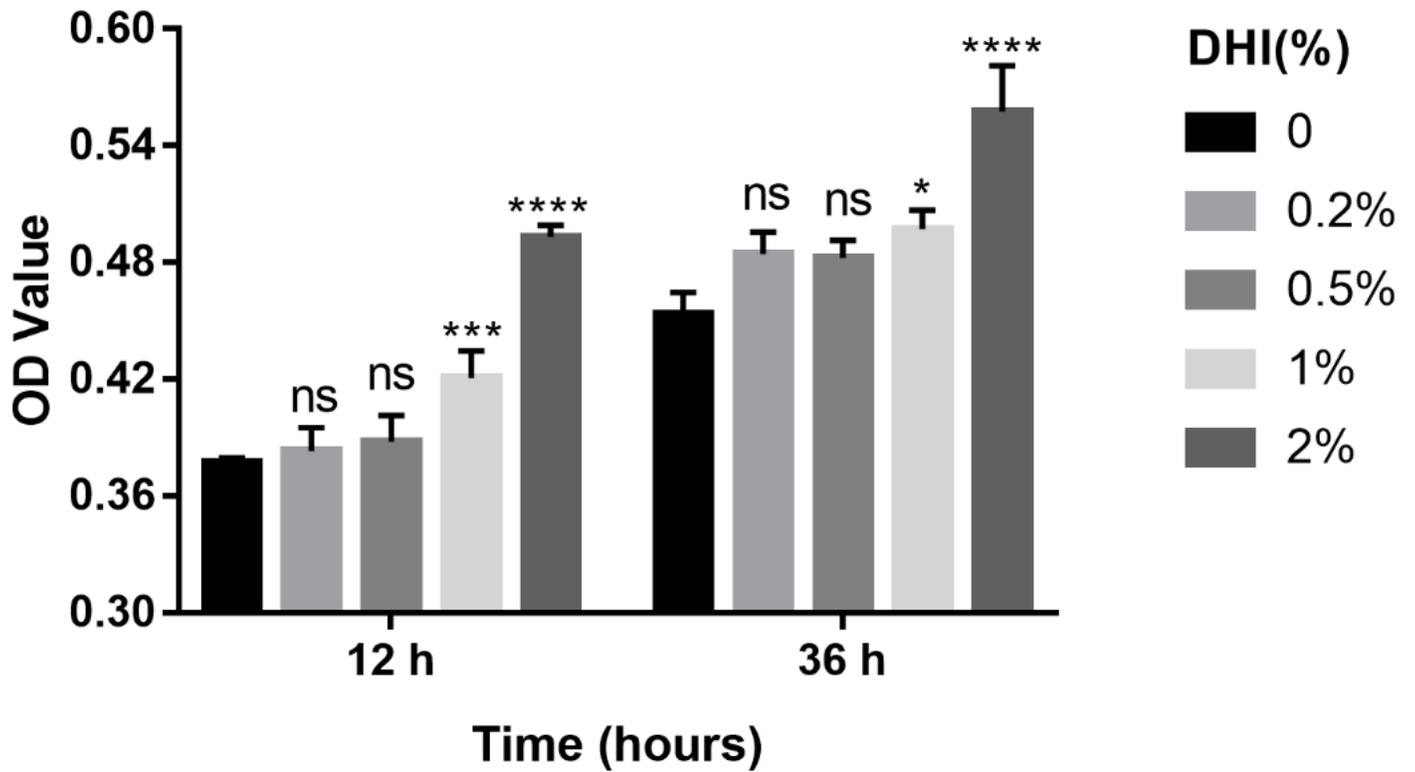
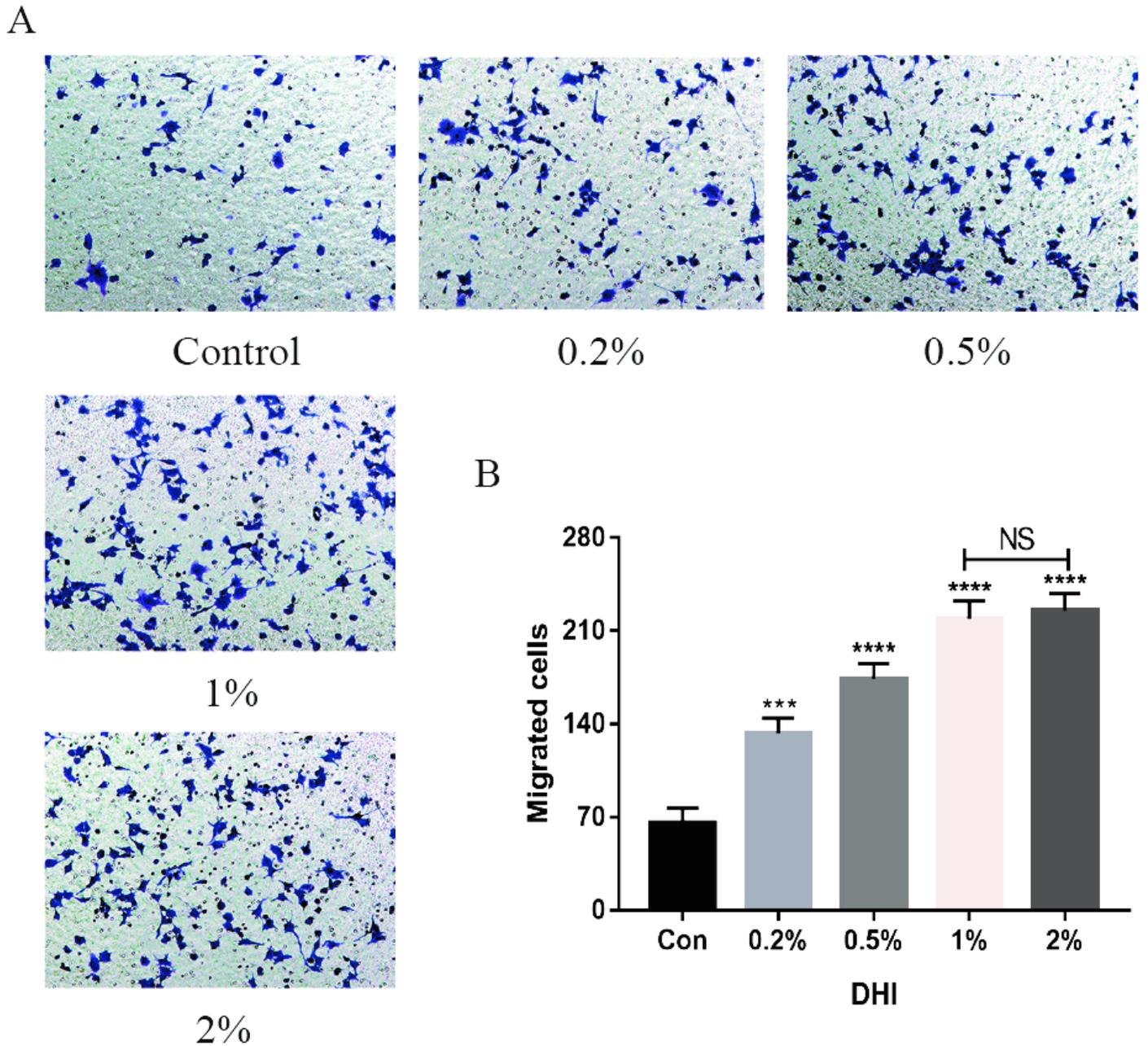


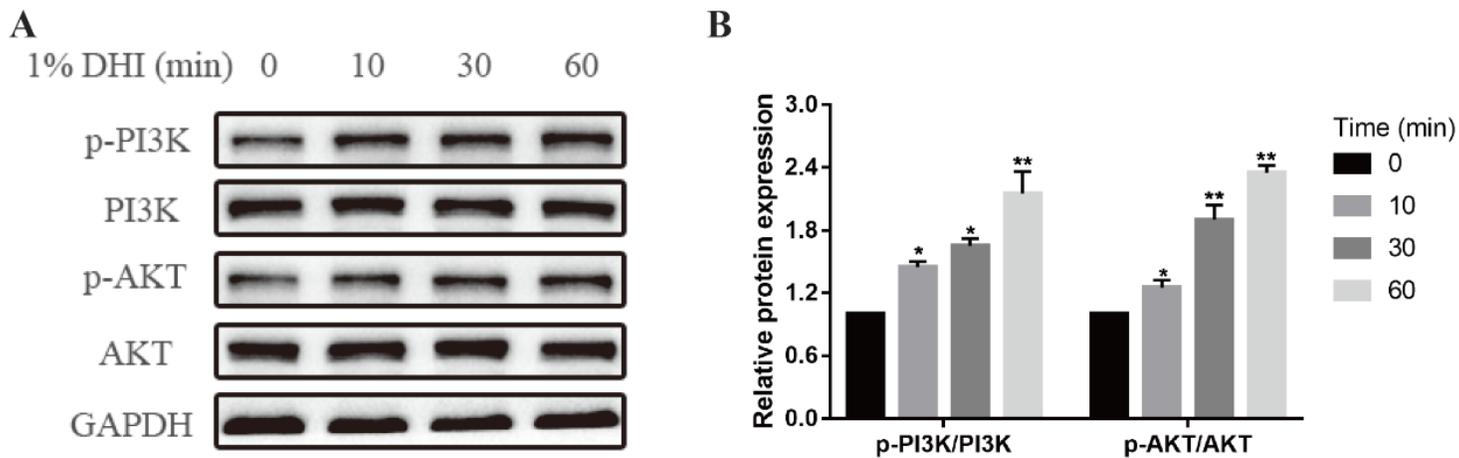
Figure 1

Effect of DHI on the proliferation of RSC96 cells. DHI had no significant effect on RSC96 cells at a low concentration (0.2%, 0.5%), but significantly promoted cell proliferation at a concentration greater than 1% in a time dependent manner. \* $p < 0.05$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to the control group.



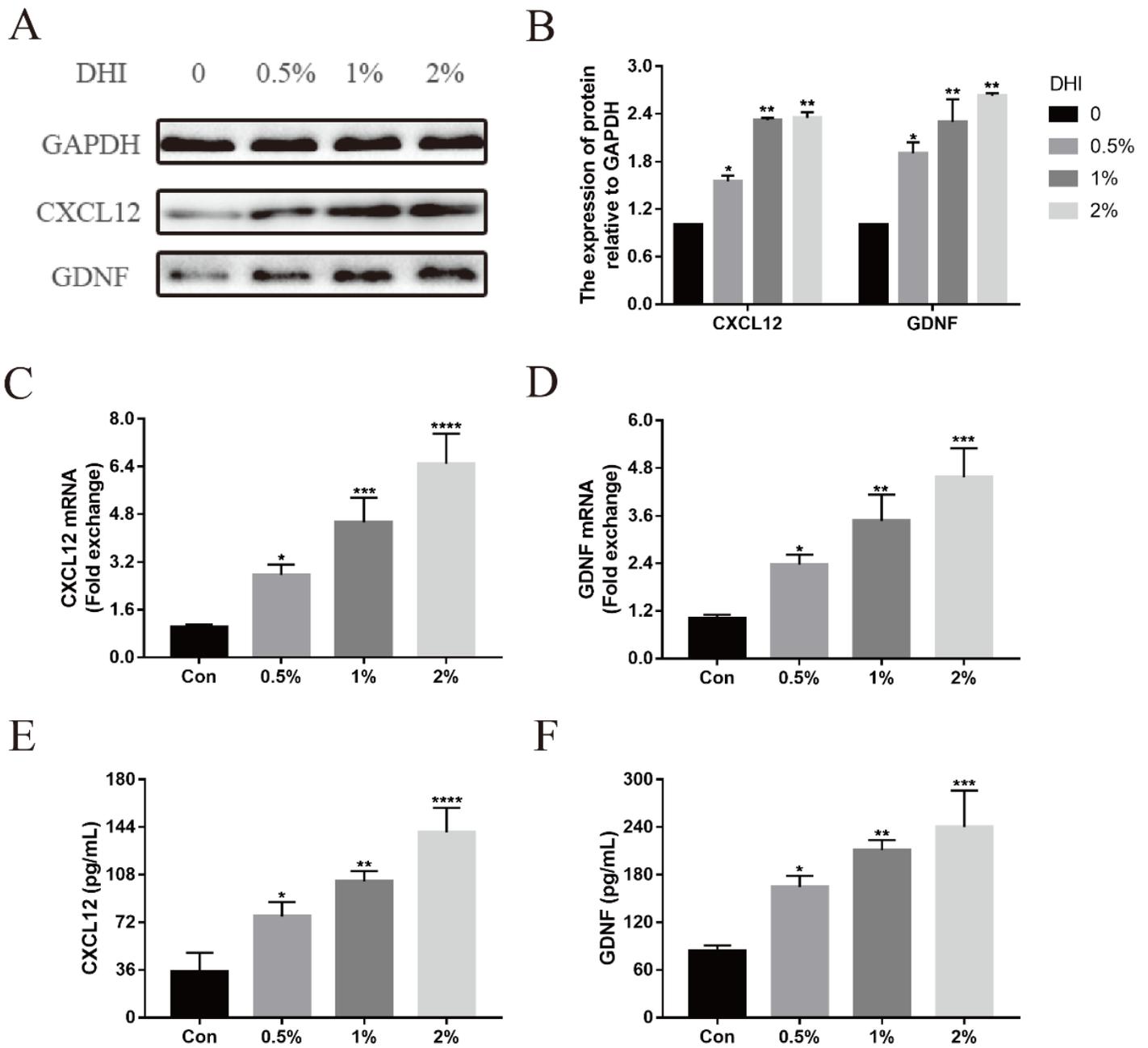
**Figure 2**

Effect of DHI on the migration of RSC96 cells. (A) The effect of DHI on the migration of RSC96 cells performed by Transwell assay. (B) Transwell qualification analysis of migratory RSC96 cells after treatment with different concentrations of DHI. \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to the control group.



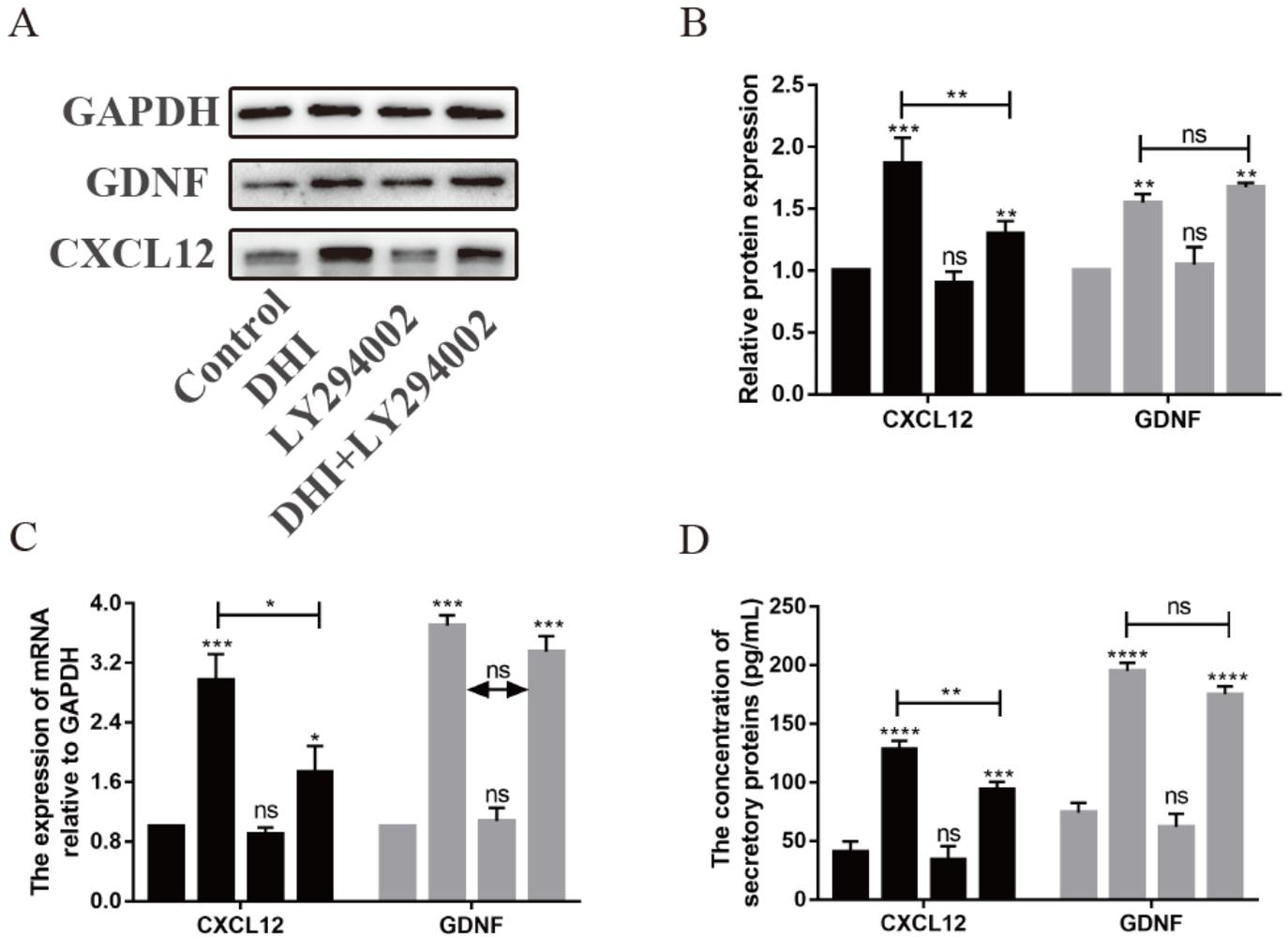
**Figure 3**

DHI activates the PI3K/AKT pathway in RSC96 cells. (A) DHI time-dependently increases the expression of p-PI3K and p-AKT, while the expression of total PI3K, AKT, and GAPDH remain unchanged. (B) The relative quantitative statistics of p-PI3K and p-AKT protein in 3A. \* $p < 0.05$ , \*\* $p < 0.005$  compared to the control group.



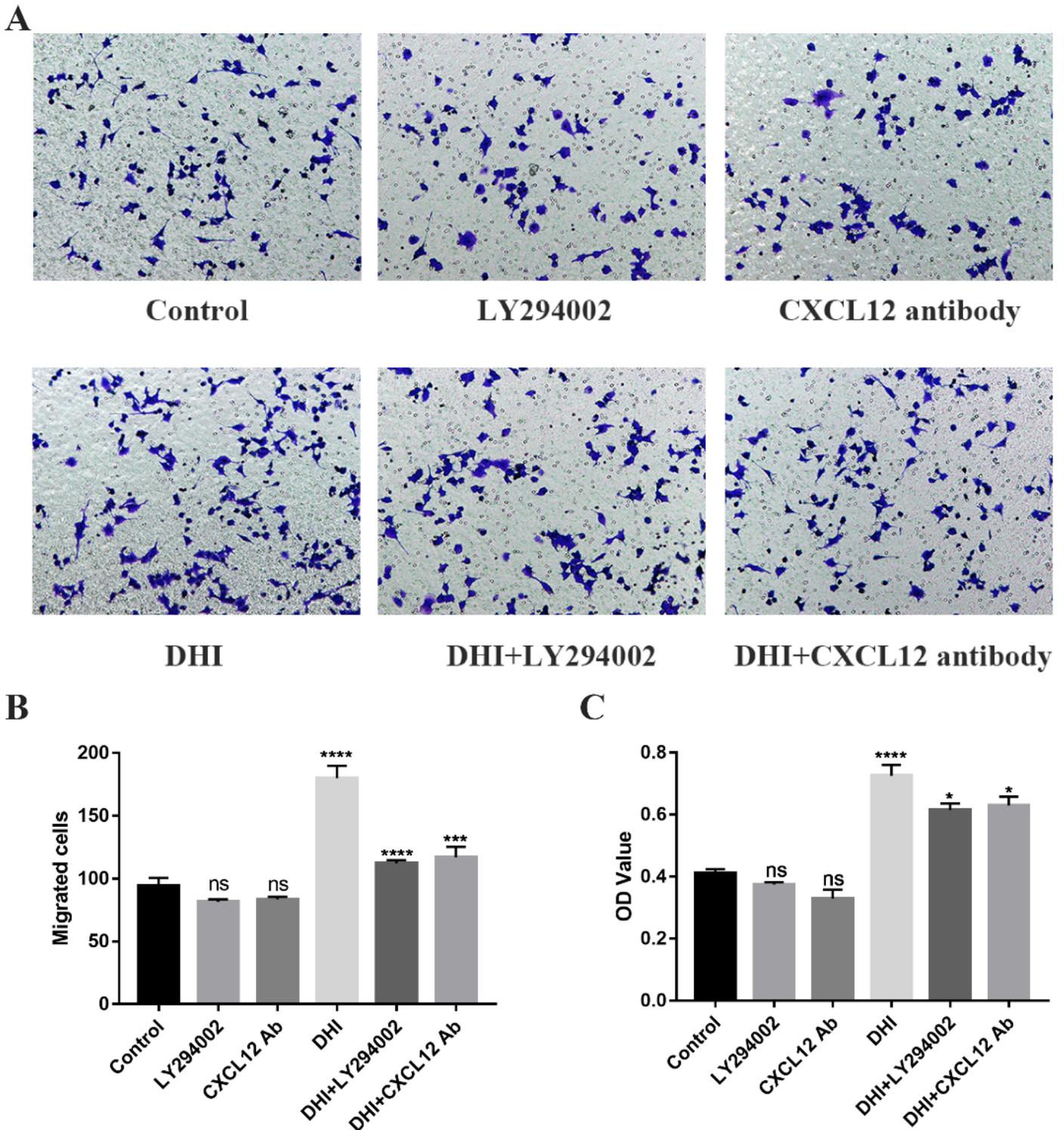
**Figure 4**

DHI increases the expression of CXCL12 and GDNF. (A) DHI concentration-dependently increases the protein expression of CXCL12 and GDNF. (B) The relative quantitative statistics of CXCL12 and GDNF protein in 4A. (C, D) DHI concentration-dependently increases the mRNA and secreted protein expression of CXCL12 and GDNF. (E, F) The relative quantitative statistics of the mRNA and secreted protein of CXCL12 and GDNF in 4C and 4D. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to the control group.



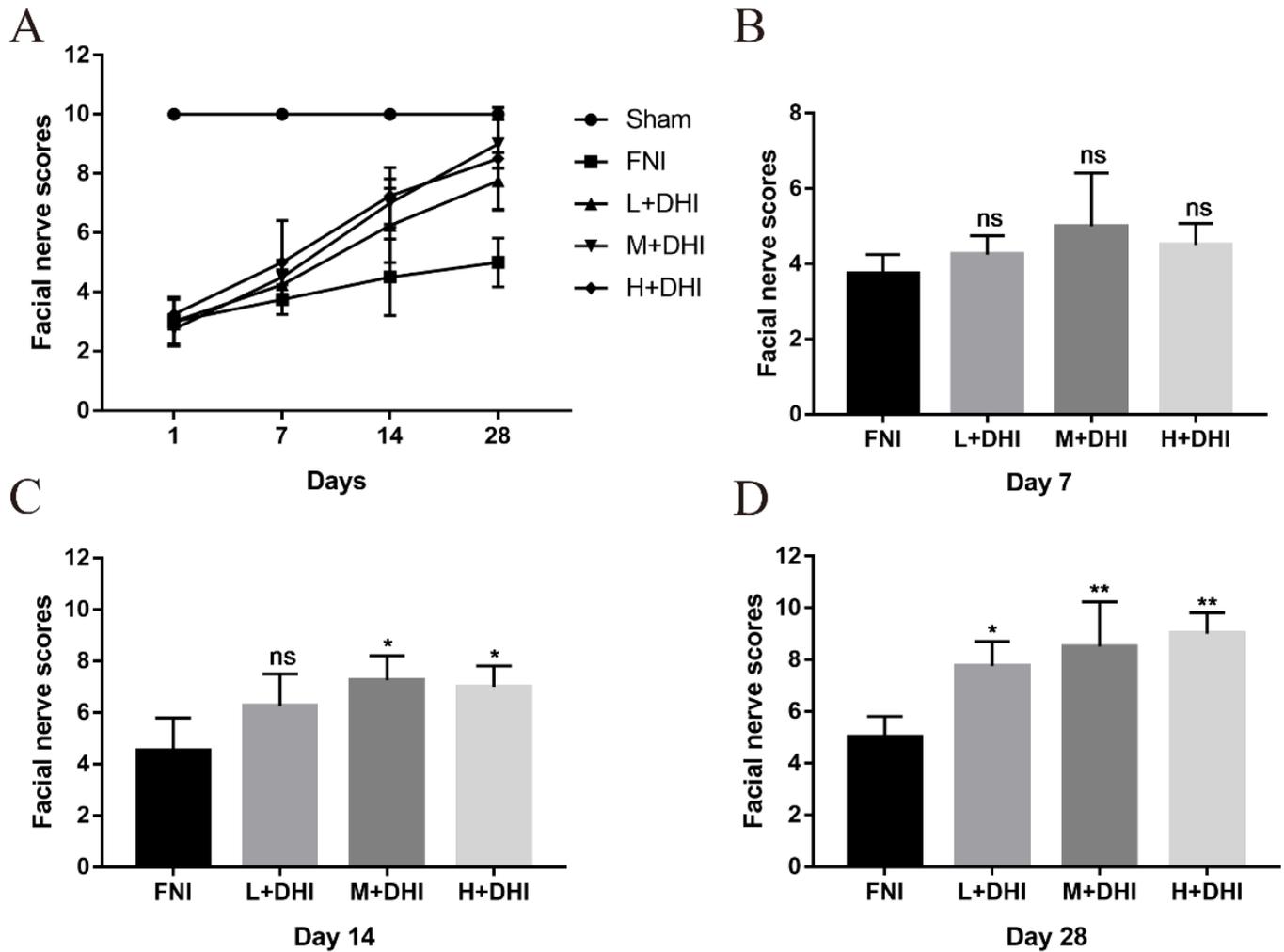
**Figure 5**

LY294002 decreases the expression of CXCL12 induced by DHI. (A, B) Effects of LY294002 on the expression of CXCL12 and GDNF proteins detected by Western Blot. (C) Effects of LY294002 on the expression of CXCL12 and GDNF mRNA detected by RT-PCR. (D) Effects of LY294002 on the secretion of CXCL12 and GDNF proteins detected by ELISA. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$ .



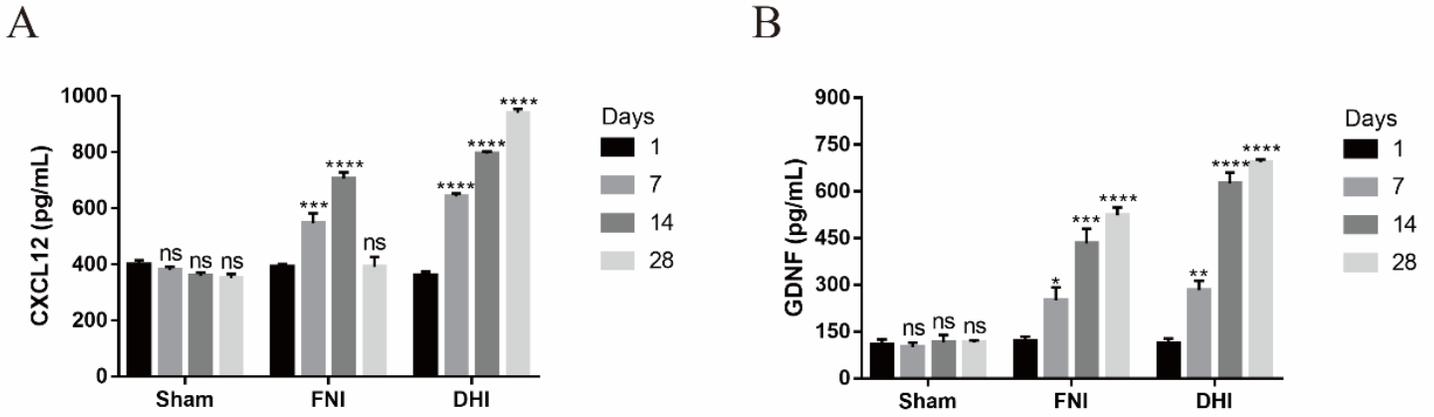
**Figure 6**

DHI promotes the proliferation and migration of RSC96 cells through PI3K/AKT pathway. (A) Effects of LY294002 and CXCL12 antibodies and their combination with DHI on the migration of RSC96 cells. (B) The quantitative statistics of RSC96 cells in 5A. (C) Effects of LY294002 and CXCL12 antibodies and their combination with DHI on the proliferation of RSC96 cells. \* $p < 0.05$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to the control group.



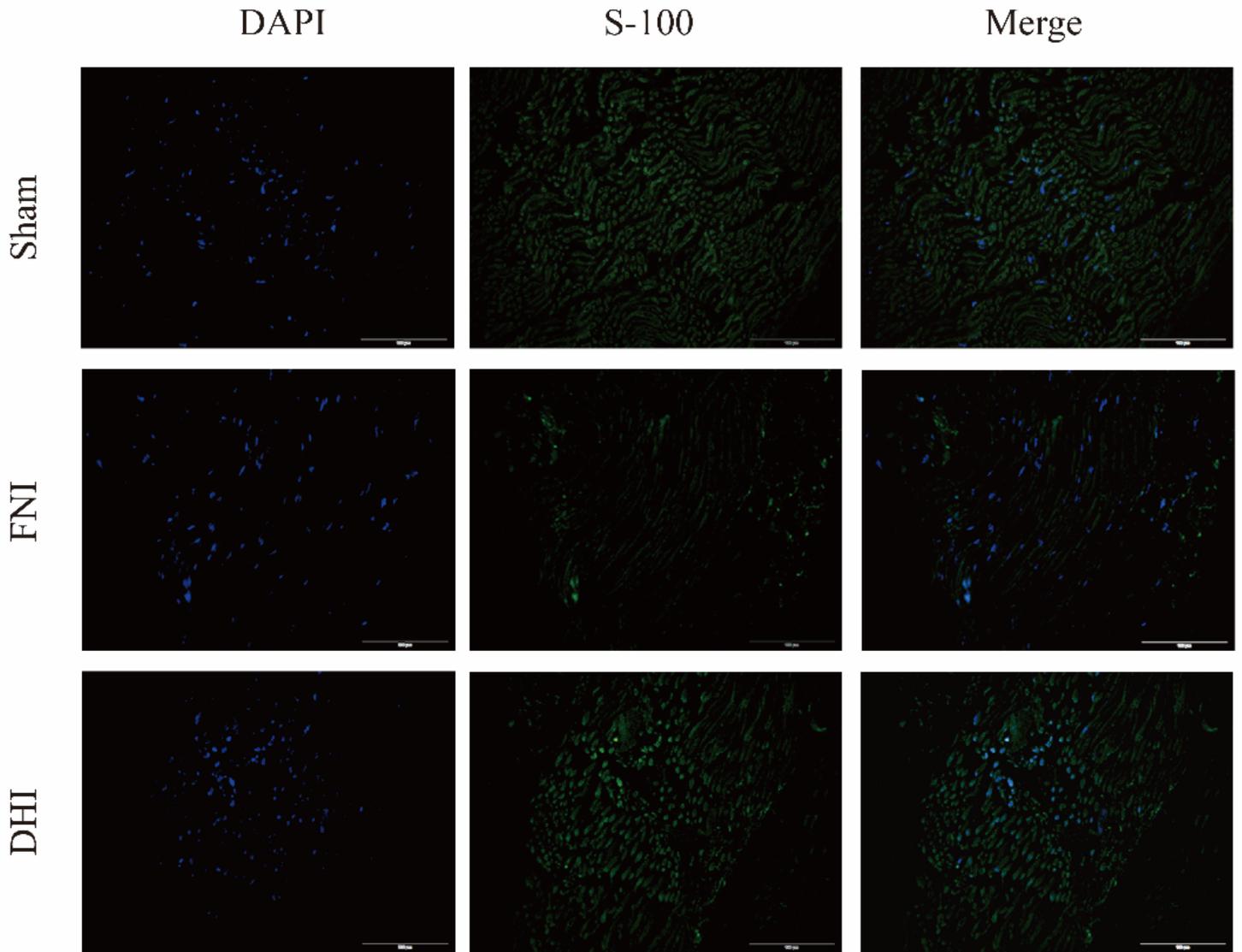
**Figure 7**

The dynamic changes of facial nerve function scores in different groups of rats after FNI. (A) Facial nerve scores of all groups at different time points. (B) The facial nerve scores of the FNI group and DHI groups on the 7th day. (C) The facial nerve scores of the FNI group and DHI groups on the 14th day. (D) The facial nerve scores of the FNI group and DHI groups on the 28th day. \* $p < 0.05$ , \*\* $p < 0.005$  compared to the FNI group.



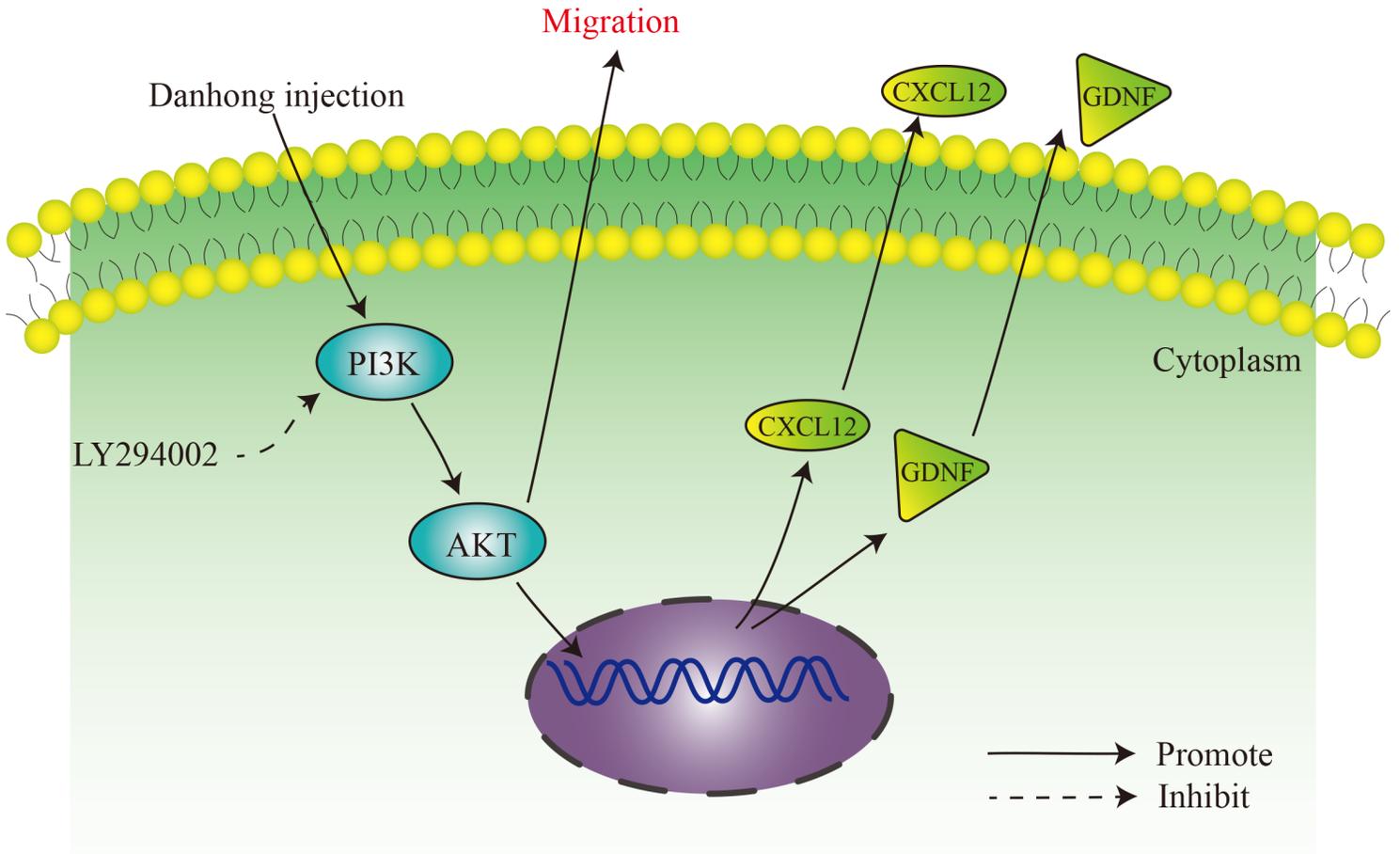
**Figure 8**

DHI increases the expression of CXCL12 and GDNF in FNI rats. (A) DHI increases the expression of CXCL12 in facial nerve-injured rats. (B) DHI increases the expression of GDNF in facial nerve-injured rats. \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to the control group.



**Figure 9**

DHI increases the expression of S-100 in the injured facial nerve. The expression of S-100 in Schwann cells was significantly decreased after facial nerve injury in the FNI group, and the expression of S-100 was significantly increased after DHI treatment.



**Figure 10**

The pattern diagram of the effect of DHI on Schwann cells.