Research Article

Buyang Huanwu decoction improves neural recovery after spinal cord injury in rats through the mTOR signaling pathway and autophagy

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Background: Spinal cord injury (SCI) refers to the interruption of the tracts inside the spinal cord caused by various factors. The repair of damaged axons has always been a difficult point in clinical treatment and neuroscience research. The treatment of SCI with Buyang huanwu decoction (BYHWD), a well-known recipe for invigorating Qi (a vital force forming part of any living entity in traditional Chinese culture) and promoting blood circulation, shows a good effect.

Methods: The rubrospinal tract (RST) transection model in rats was established in this study and rats were administrated with low (BL), medium (BM), or high (BH) doses of BYHWD.

Results: Compared with the SCI group, BL, BM moderately, and BH significantly improved the motor function of forelimbs and increased the number of red nucleus neurons in SCI rats. As for the possible molecular mechanism, BL, BM moderately, and BH significantly increased mTOR whereas decreased Beclin-1 and LC3 in the red nucleus.

Conclusion: In conclusion, low, medium, and high doses of BYHWD could promote neural recovery in SCI rats through improving motor function and neuron survival in the red nucleus. The neuroprotective effects of BYHWD might be associated with affecting the mTOR signaling pathway and autophagy.

Keywords: Spinal cord injury (SCI), Buyang Huanwu decoction (BYHWD), Rubrospinal tract (RST), Red nucleus, mTOR signaling pathway, Autophagy

Introduction

Spinal cord injury (SCI) refers to the interruption of the tracts inside the spinal cord caused by various factors, affecting the transduction of nerve signals in ascending and descending tracts, resulting in the loss of corresponding body movement and sensory function. SCI often causes severe dysfunction of limbs below the injured segment.[1] The repair of damaged axons has always been a difficult point in clinical treatment and neuroscience research.

In traditional Chinese medicine (TCM), hemiplegia, paraplegia or limb atrophy caused by SCI is considered

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to be damage to the Du meridian, resulting in blood stasis and collateral obstruction, as well as severe damage to the patient's vital energy, causing Qi deficiency. It is reported that the treatment of SCI with Buyang Huanwu decoction (BYHWD) has a good effect. BYHWD is a well-known recipe for invigorating Qi and promoting blood circulation, with the alkaloids, glycosides, polysaccharides, and aglycones in the formula as its main effective parts.[2] BYHWD has been reported to prevent axotomy-caused neuron death and atrophy in red nucleus, promote the regrowth of axons and enhance the recovery of forelimb function.[3] BYHWD could lead to the motor function recovery of limbs in SCI rats and its neuroprotection role showed to be related to the regulation of apoptosis-associated protein expression.[4] Moreover, a combination of BYHWD treatment with the transplantation of bone marrow mesenchymal stem cells (BMSCs) or embryonic neural stem cells exerted a better neuroprotective effect on the red nucleus neurons following SCI.[5,6] BYHWD has been used in China to treat SCI for centuries, and it has been proven to improve the prognosis of SCI by clinical trials; however, the mechanisms remain unclear.

SCI is a serious type of nerve injury that can induce autophagy in neurons. Appropriate levels of autophagy can play a protective role at the early stage of acute injury; however, excessive autophagy will aggravate the injury. In a model of traumatic brain injuries, the autophagy inhibitor of chloroquine inhibited autophagy, decreased the expression levels of inflammatory factors such as TNF-α and IL-1β, reduced brain edema, and increased neural function recovery, playing a neuroprotective role.[7] Mammalian target of rapamycin (mTOR)is a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases. It plays as a serine/threonine-protein kinase, which regulates cell growth, metabolism, protein synthesis, autophagy, and transcription.[8] Besides, the mTOR modulates the regeneration process of damaged nervous tissue. As previously reported, Akt and mTOR could be inhibited by upstream phosphatase and tensin homolog (PTEN) and the loss of either PTEN or tuberous sclerosis complex 1 (TSC1) would lead to constitutive activation of mTOR and exert significant neuroprotection and axon growth-promoting effects upon damaged retinal ganglion cells.[9,10] Bisperoxovanadium compounds are potent PTEN inhibitors, which are shown to exert neuroprotection functions on a variety of central nervous system (CNS) injury/disease models.[11–13] Inhibition of PTEN by bisperoxovanadium could lead to neuroprotective effects and recovery of function after cervical unilateral contusive SCI.[14] Moreover, bisperoxovanadium markedly promoted downstream Akt/mTOR signaling pathway and decreased autophagy, suggesting that inhibition of PTEN may mediate the effects of bisperoxovanadium in SCI.[14] Thus, we hypothesize that BYHWD might improve the outcome function of SCI through mTOR signaling pathway and autophagy-related manners.

In the current investigation, we employed the rubrospinal tract (RST) transection model to assess the role of BYHWD in forelimb motor function and red nucleus neuron apoptosis in SCI rats. The changes of mTOR and autophagy-related factors in the red nucleus of different groups of rats were detected. The neuroprotective effect of BYHWD was investigated to provide an experimental basis for further exploring the mechanism of treating SCI by TCM.

Materials and methods

Ethics

All animal experiments complied with the Guidelines for the Care and Use of Experimental Animals and were approved by the Experimental Committee of the First Affiliated Hospital of Hunan University of Chinese Medicine.

Animals

Sixty adult male Sprague–Dawley (SD) rats (wt 180–220 g; aged 8–9 weeks) were provided by the Laboratory Animal Center of Hunan University of Chinese Medicine (license number SYXK [Xiang] 2016-0005; Changsha, China). Rats were kept separately in plastic cages at 22–25°C in a relative humidity of 50–70%. Rats were housed under a constant light–dark cycle and were allowed free access to food and water.

Grouping and experimental design

Rats were randomly divided into the control group, SCI model group, BYHWD low/medium/high dose (BL/BM/BH)groups using a computer random number method. Except for the control group, rats in the remaining four groups received RST transection for SCI model establishment. Rats in BL, BM, and BH groups were treated with a corresponding dose of BYHWD (6.41, 12.82, and 25.65 g/kg/d) for 4 weeks by intragastric administration, respectively. Penicillin (40,000 international units for 5 d) was injected subcutaneously to prevent infection. The behavior of rats in each group was evaluated by the inclined plate test before RST transection and on day 1, 14, and 28 after the operation. After the final behavior evaluation, all

rats were anesthetized and brain tissue (the red nucleus) was collected on day 29. The rats used for the immunofluorescence staining and Nissl's staining were intracardially perfused with 4% paraformaldehyde according to a fixation protocol; the remaining rats were kept and the fresh red nucleus of the left side was dissected for real-time qPCR detection. The experimental design is shown in Fig. 1A.

RST transection procedures

RST transection model is often used in SCI repair research because of its clear bundle, simple operation, and small trauma. The RST transection operation was performed following the methods described before.[3] Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (30 mg/kg), the cervical curvature was fixed in the prone position, and the soft tissue and superficial muscles were separated under the operating microscope. Part of the erector spinae attached to the right side of the C2 spinous process was removed to expose the ligamenta flava between the C3 and C4 vertebral arches, and the ligamenta flava was cut open to expose the spinal cord. A small incision was made through the dura mater following identifying the dorsal root of the spinal nerve. Then, the right dorsolateral funiculus of the spinal cord was transected using a No. 12 surgical blade. This operation completely transected the lateral funiculus (containing the RST) and partially injured the ipsilateral ventral funiculus and gray matter, leaving the dorsal columns intact. When the animal woke up, the right forelimb was flexed and close to the trunk, with uncoordinated movement, and the right forepaw could not open, indicating the success of the operation.

BYHWD formula and treatment

BYHWD was formulated as previously described.[5,15,16] Briefly, it is composed of *Astragalus membranaceus (Fisch.) Bunge* 120 g, *Angelica sinensis (Oliv.) Diels* 6 g,*Paeonia lactiflora Pall.* 4.5 g, *Ligusticum chuanxiong Hort.* 3 g, *Pheretima aspergillum (E. Perrier)* 3 g, *Prunus davidiana (CarriŠre) Franch.* 3 g and *Carthamus tinctorius L.* 3 g. All BYHWD herbal components (from GAP planting base) were purchased from the LBX Pharmacy Co., Ltd (Changsha, Hunan province, China) and were identified by experts in the School of Pharmacy, Hunan University of Chinese Medicine. The mixture was decocted in boiling water for 45 min, concentrated and vacuum-dried to form a paste, containing 2 g crude extracts per gram. This BYHWD paste was diluted with distilled water to a concentration of 1 g/ml, which was stored at 4°C for further use. The BL, BM, and BH groups were treated with BYHWD by intragastric administration at doses of 6.41, 12.82, and 25.65 g/kg, respectively, once a day for 28 d. The control group was treated with an equal amount of normal saline.

Motor function evaluation

The recovery of motor function in rats was evaluated using the inclined plate test on the time points before RST transection (day 0), and 1, 14, and 28 d after the operation following the methods described before.[17] The rat was placed on the inclined plate with the body axis perpendicular to the inclined plate and the residence time was measured. The inclined plate test was started from 0°. After 5 s, the angle of the inclined plate was increased by 5° each time. The maximum angle at which the rat could stay for 5 s was recorded as the corresponding functional value. Each rat was measured 3 times and the highest angle was recorded.

Brain tissue sample collection

On day 29 after the RST transection (1 d after the last motor function evaluation), 6 rats in each group were anesthetized and intracardially perfused with 4% paraformaldehyde. The brain was taken off immediately; the cerebrum and cerebellum were removed. Only the brain stem was kept and the red nucleus was located anterior to the superior colliculus. Samples were embedded in optimal cutting temperature compound, sliced at a thickness of 20 μm with a constant freezing microtome at −20°C. The sections were applied for immunofluorescence and Nissl's staining. The rest of the rats were anesthetized and the fresh red nucleus of left side were collected for real-time qPCR detection.

Immunofluorescence staining

Tissue sections were fixed in paraformaldehyde solution at 37°C for 30 min, washed twice with PBS, and repaired with citrate antigen. After blocking the tissue sections in 10% goat serum at 37°C for 40 min, proper primary antibodies, anti-mTOR (ab32028, abcam, dilution, 1:400), anti-Beclin-1 (ab210498, abcam, dilution, 1: 200),and anti-LC3 II (ab192890, abcam, 1: 400) were added, respectively. Sections were incubated at 4°C overnight. After washing with PBS three times (5 min each time), sections were incubated with Alexa Fluor 488 or Alexa Fluor 594 labeled Donkey Anti-Rabbit IgG (1: 200). Then, sections were placed in a wet box at 37°C for 1 h, washed with PBS three times (5 min each time), added with 50-100μl DAPI, placed in the dark for 10 min, and then mounted. Five fields were selected for each slice in the same area, and the

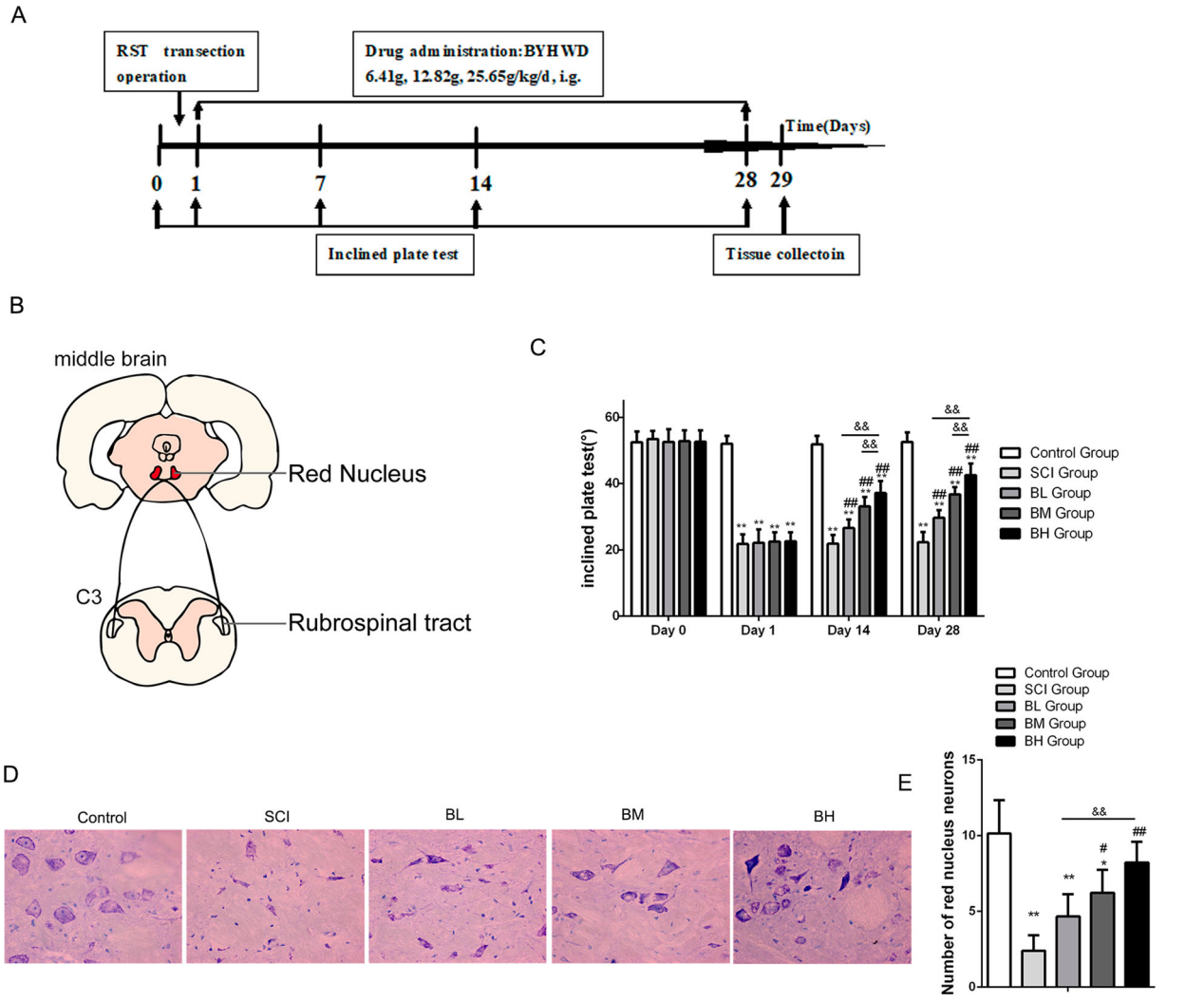

Figure 1 Drug administration and motor function evaluation (A) A schematic diagram showing the experimental design. A total of 60 rats were randomly divided into 5 groups ($n = 12$): the control, SCI, BYHWD low dose (BL), BYHWD medium dose (BM), and BYHWD high dose (BH) groups. SCI model was established using RST transection operation. Rats in BL (6.41 g/kg/d), BM (12.82 g/kg/d), and BH (25.65 g/kg/d) groups received treatment with BYHWD by intragastric administration for 4 weeks. Inclined plate test and tissue sample collection were performed at the indicated time points. (B) Schematic diagram showing the midbrain at the level of the red nucleus and the 3rd cervical segment. The RST transection operation was performed between the level of C3 and C4. (C) Results of inclined plate test in each group on time points before RST transection (day 0) and day 1, 14, and 28 after operation. (D–E) The numbers of red nucleus neurons were examined by Nissl's staining and quantitatively analyzed. **P < 0.01, compared with the control group; #P < 0.05, ##P<0.01, compared with the SCI group; &&P < 0.01, compared with the BH group

ratio of single-positive cells in each field was counted by Image Pro Plus.

Nissl's staining

Nissl's staining was performed on tissue sections as described in our previous study.[9] Red nucleus neurons were observed under a biological imaging microscope (Olympus, Tokyo, Japan). Red nucleus neurons with identifiable nuclei, nucleoli, and characteristic neuronal morphology were counted.

Real-time quantitative polymerase chain reaction (real-time qPCR)

Cells were digested and lysed, and total RNA was extracted. A total of 5 μl of RNA was obtained from each group, and ultra-pure water of RNase was added to dilute the substance at a ratio of 1:20. NanoPhotometer ultramicro-ultraviolet spectrophotometer (Biorad, Hercules, CA, USA) was used to measure OD260/280 values for calculating the RNA concentration and purity. The OD 260/280 ratio was kept between 1.8 and 2.0 to ensure RNA purity.

Agarose gel electrophoresis (1%) was performed to maintain integrity. The reverse transcription was performed according to the Fermentas kit instructions. Amplification reactions were performed using the Universal SYBR Green Master system (Roche, Basel, Switzerland). Amplification conditions were as follows: denaturation at 95°C for 2 min, denaturation at 95°C for 10 sec, annealing at 57°C for 10 sec, extension at 72°C for 15 sec, a total of 40 cycles of denaturation, annealing and extension. Primer 5.0 software was used for primer design and the primer sequences are shown in Table S1. β-actin (Sangon Biotech, Shanghai, China) was used as an internal control Ct and $2^{-\Delta\Delta Ct}$ were obtained and used for quantitative analysis of Beclin1, LC3, and mTOR mRNA expression.

Statistical analysis

SPSS 24.0 software (Chicago, IL, USA) was used for statistical analysis. The results were expressed as mean ± SD. All data were subject to normality test and variance homogeneity test. LSD test was used for the homogeneity of variance; otherwise, Dunnett's T3 method was used. If the normality test is not satisfied, the rank sum test is used. A one-way ANOVA was performed to analyze the differences. Repeated measurement analysis of variance was used to analyze the results of inclined plate experiments. $P < 0.05$ indicated a statistically significant difference.

Results

Drug administration on SCI rats and motor function evaluation

As shown in the schematic diagram (Fig. 1A), we randomly divided a total of 60 rats into 5 groups ($n = 12$): the control, SCI, BL, BM, and BH groups. Rats in each group were treated accordingly. The inclined plate test was performed to evaluate the motor function of the forelimbs. The schematic diagram of red nucleus in the midbrain and RST was shown in Fig. 1B. As shown in Fig. 1C, 1D after RST surgery, compared with the control group, the inclined plate test scores of the SCI, BL, BM, and BH groups were significantly reduced ($P < 0.01$); on day 14 and 28 after RST surgery, compared with SCI group, the inclined plate test scores of BL, BM, and BH groups were higher ($P < 0.01$), and the score of BH group showed to be higher than those of BL and BM groups ($P < 0.01$). On day 29 after the RST surgery, we collected the red nucleus tissues from animals in each group and performed Nissl's staining to examine the numbers of red nucleus neurons. Representative images are shown in Fig. 1D. The statistical analysis of the red nucleus neurons number was shown in Fig. 1E. As compared to the control group, the numbers of red nucleus neurons decreased in SCI ($P < 0.01$), BL ($P < 0.01$), BM ($P < 0.05$), and BH groups. As compared to the SCI group, the number of red nucleus neurons was higher in BL, BM ($P < 0.05$), and BH ($P < 0.01$) groups. The BH group showed higher red nucleus neurons than the BL group ($P < 0.01$). These data indicated that all of the BYHWD groups (BL, BM, and BH) improved the neural recovery in SCI rats, among which BH treatment showed a better effect.

Changes of mTOR, LC3, and Beclin-1 in the red nucleus

After SCI, single-labeled immunofluorescence staining showed that a small number of mTOR-positive cells (red fluorescence; Fig. 2A) but a large number of Beclin-1- (green fluorescence; Fig. 2B) and LC3-positive cells (green fluorescence; Fig. 2C) were seen in the red nucleus tissue of the SCI group, in comparison with the control group. As compared to the SCI group, the rate of the mTOR-positive cells showed to be dramatically increased in BL, BM, and BH groups ($P < 0.01$; Fig. 2A), and the rate of the mTOR-positive cells was higher in the BH group compared with BL ($P < 0.01$; Fig. 2A) and BM ($P < 0.01$; Fig. 2A) groups. On the contrary, in comparison with SCI group, the rate of the Beclin-1- ($P < 0.01$; Fig. 2B) and LC3-positive cells ($P < 0.01$; Fig. 2C) was significantly lower in BL, BM, and BH groups, and the rate of the Beclin-1- and LC3-positive cells was lower in BH group compared with BL ($P < 0.01$; Fig. 2B and C) and BM ($P < 0.01$; Fig. 2B and2C) groups.

Similarly, mTOR, LC3, and Beclin-1 mRNA expression in the red nucleus in each group were examined by real-time PCR. Compared with SCI group, mTOR mRNA expression was significantly higher in BL, BM, and BH groups ($P < 0.05$ or <0.01; Fig. 3), and mTOR mRNA expression was higher in the BH group compared with BL and BM groups ($P < 0.05$ or <0.01; Fig. 3). Conversely, compared with SCI group, Beclin-1 and LC3 mRNA expression was significantly lower in BL, BM, and BH groups ($P < 0.05$ or <0.01; Fig. 3), and Beclin-1 and LC3 mRNA expression was lower in BH group compared with BL and BM groups ($P < 0.05$ or <0.01; Fig. 3). In summary, the treatment of different doses of BYHWD might activate the mTOR signaling pathway whereas inhibiting the autophagy following SCI.

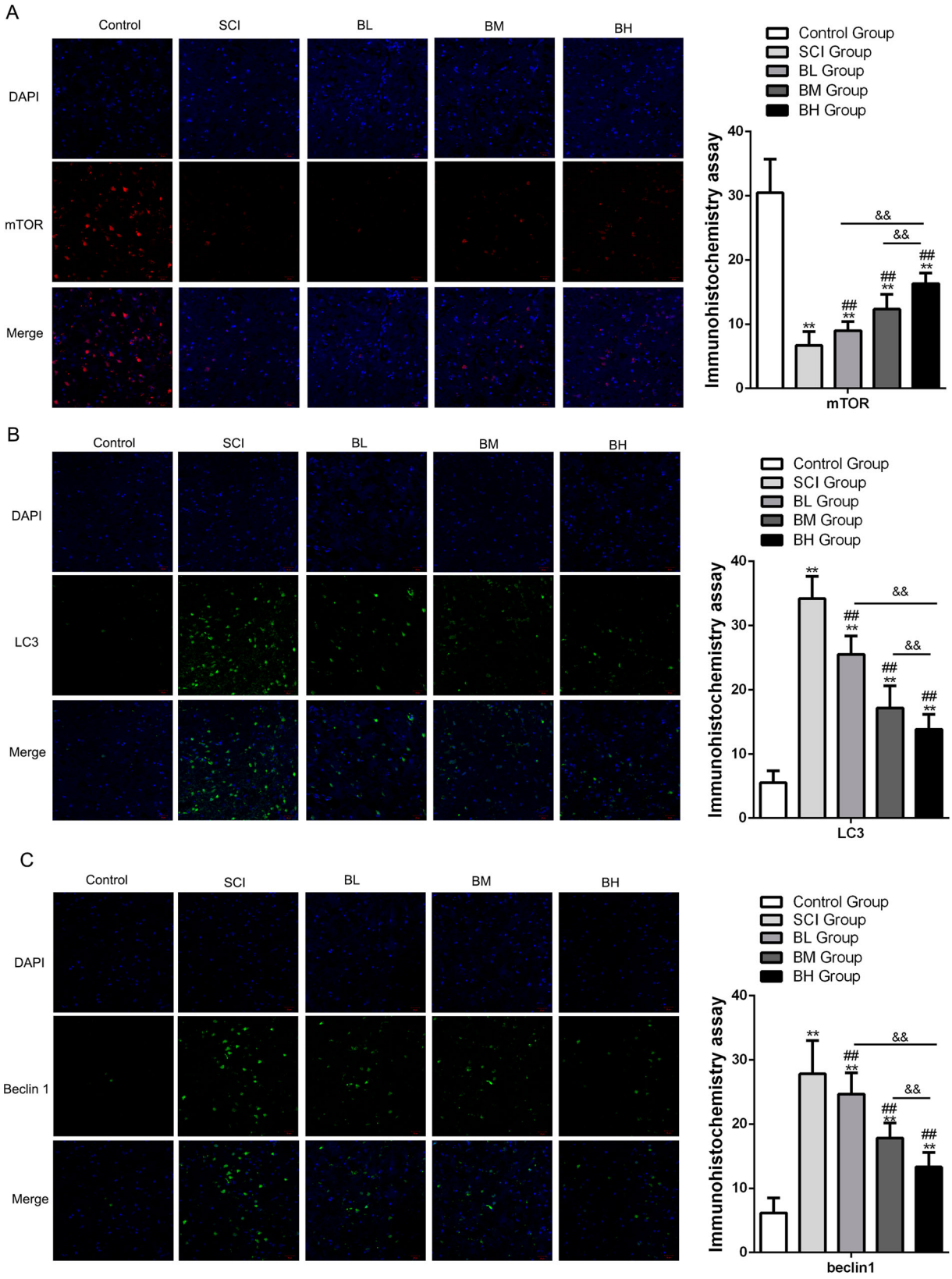

Figure 2 Expression of mTOR, LC3, and Beclin-1 protein in red nucleus On day 29 after the RST transection, red nucleus tissues were detected for the expression of (A) mTOR (red fluorescence), (B) LC3 (green fluorescence), and (C) Beclin-1 (green fluorescence) using immunofluorescence staining. Red or blue fluorescence represented the corresponding protein existing in the red nucleus neurons. **P < 0.01, compared with the control group; ##P < 0.01, compared with the SCI group; &&P < 0.01, compared with the BH group

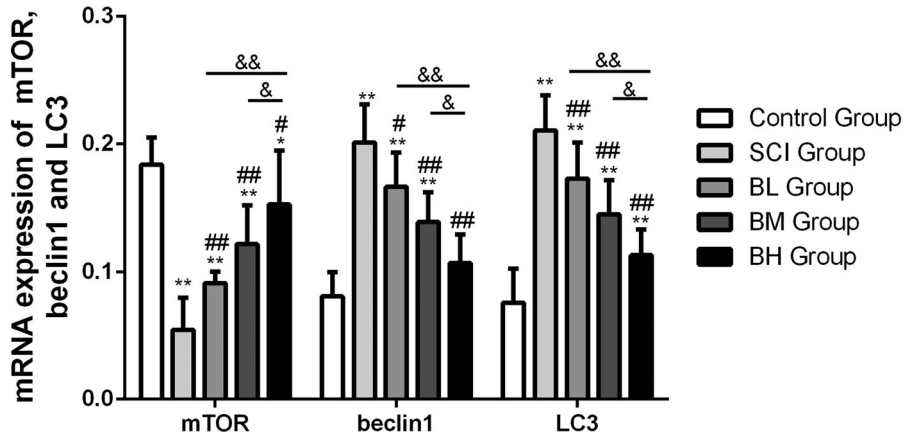

Figure 3 Changes of mTOR, LC3, and Beclin-1 mRNA expression in red nucleus On day 29 after the RST tansection, red nucleus were collected and examined for the mRNA expression of mTOR, LC3 and Beclin 1. **P < 0.01, compared with the control group; #P < 0.05, ##P < 0.01, compared with the SCI group; &P < 0.05, &&P < 0.01, compared with the BH group

Discussion

In the present study, we demonstrated the effect of BYHWD treating SCI. Specifically, we showed that low, medium or high doses of BYHWD mediate different responses. Compared with the SCI group, BL, BM moderately, and BH significantly improved the motor function of forelimbs and increased the red nucleus neurons number in SCI rats. As for the possible molecular mechanism, BL, BM moderately, and BH significantly increased mTOR whereas decreased Beclin-1 and LC3 in the red nucleus.

In TCM, SCI can be classified into Qi-deficiency and blood-stasis syndrome.[5] Based on its theories, the dysfunction caused by CNS injury, such as hemiplegia, deviation of mouth and eyes, dysphasia, slobbering, *etc.*, is mostly due to the damage of the DU meridian, which leads to Qi-deficiency and blood-stasis, obstruction of channels and collaterals, and muscle hypotrophy.[18] In terms of treatment, since ancient times, doctors have mostly used the method of invigorating Qi and activating blood, nourishing the body and removing pathogenic factors, eliminating blood stasis and unblocking collaterals. The main ingredients of BYHWD can restore vital energy, invigorate the blood, remove blood stasis and regulate the collaterals; thus, BYHWD was originally used for treating stroke with Qi-deficiency and blood-stasis syndrome and has been found to have a good effect on SCI recently.[3–6,15,16] In our previous study, we found that BYHWD combined with BMSCs transplantation had a synergistic effect on neuroprotection of red nucleus neurons after SCI, which may be associated with the cAMP/CREB pathway.[5]

In the present study, we established the RST transection model in rats. One day after the RST transection operation, the inclined plate scores in all groups were significantly reduced, and the neurons number in the red nucleus also decreased, indicating the successful modeling. Herein, the study demonstrated that all of the three doses of BYHWD improved the motor function and the neuron survival in the red nucleus to some degree; of the three doses, BH had the best improving effects. These data suggest that BYHWD plays a neuroprotective role after SCI, improving the neuron survival in the red nucleus tissues and promoting the motor function recovery of limbs.

It is generally accepted that activating PI3K/Akt signaling pathways with downstream mTOR protein complexes helps prevent cellular death and enhance cell processes related to growth and proliferation. After SCI, the deletion of PTEN or its function promoted the activity of downstream PI3K/Akt and mTOR signaling pathways, enhanced axonal regeneration after SCI,[19] decreased the atrophy of motor neurons,[20] and improved nerve survival.[13,21] Normally, PI3K/AKT signaling downstream mTOR is inactivated in the neurons of the spinal cord.[22] However, drugs like bisperoxovanadium significantly promoted mTOR signaling pathway and inhibited autophagy, thus enhanced strong neuroprotective effects by decreasing the death of motor neurons, promoting tissue sparing, reducing cavity formation, and improving forelimb motor function in rats.[14] Similarly, in the present study, BL, BM moderately, and BH significantly increased mTOR mRNA expression and protein levels, suggesting that BYHWD might exert its neuroprotective effects through mTOR signaling pathway.

Notably, mTOR is a direct target of rapamycin, an autophagy agonist. As we have mentioned, excessive autophagy might aggravate the injury. Autophagy is thought to be important for the turnover of whole organelles and long-lived proteins. In response to cellular or

nutrient stress, autophagy could be elevated for energy acquisition. Nevertheless, the dysregulation of autophagy is found to result in autophagic, or Type II programmed cell death after SCI.[23,24] Because mTOR is known for its regulatory function of autophagy, herein, we also investigated the changes of autophagy markers, Beclin-1 and LC3. Consistent with previous studies, BL, BM moderately, and BH significantly downregulated Beclin-1 and LC3 mRNA and protein expression. These data suggest that BYHWD could activate mTOR and inhibit autophagy in the red nucleus after SCI.

In conclusion, low, medium, and high doses of BYHWD could promote neural recovery in SCI rats through improving motor function and neuron survival in the red nucleus. The neuroprotective effects of BYHWD might be associated with affecting the mTOR signaling pathway and autophagy.

Disclaimer statements

Contributors None.

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Conflicts of interest None.

Ethics approval

All animal experiments complied with the Guidelines for the Care and Use of Experimental Animals and were approved by the Experimental Committee of the First Affiliated Hospital of Hunan University of Chinese Medicine.

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