Research article

Neuroprotective effects of the Buyang Huanwu decoction on functional recovery in rats following spinal cord injury

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Background: The Buyang Huanwu decoction (BYHWD) is a traditional Chinese herbal prescription and has been used in China to treat spinal cord injury (SCI) for hundreds of years. Clinical trials have shown that BYHWD improves the outcome of SCI in clinical trials, but the mechanisms are not known. This study observed the neuroprotective effects of BYHWD on spinal nerve cells after SCI and investigated possible mechanisms.

Materials and methods: Forty female Wistar rats were randomized equally to four groups treated by sham injury, SCI, BYHWD, or methylprednisolone (MP). The Basso, Beattie, and Bresnahan (BBB) score was used to evaluate hind-limb locomotor function. Neuron apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling staining and caspase-3, Bax, and Bcl-2 mRNA and protein expression were evaluated by real-time quantitative polymerase chain reaction and Western blotting, respectively.

Results: In the sham group, walking was mildly abnormal after anesthesia but recovered completely in 2 days. The BBB score in the SCI model group was significantly different from that in the sham group. The BBB scores of rats in both the BYHWD and MP groups were significantly higher than scores of rats in the SCI group. BYHWD had an antiapoptosis effect, as shown by significant decreases in expression of caspase-3 and Bax and increase in Bcl-2 expression.

Conclusion: BYHWD treatment restored hind-limb motor function of rats with SCI. The neuroprotective effect of BYHWD was associated with modulation of the expression of apoptosis-related proteins.

Keywords: Spinal cord injury, BYHWD, Nerve cells, Apoptosis, Animal model

Introduction

Spinal cord injury (SCI) generally results in permanent disabilities with high healthcare costs and heavy social and psychological burdens.[1] Primary injury of spinal cord tissue caused by trauma and secondary injury caused by apoptosis, hemorrhage, edema, inflammation, excitotoxicity, and oxidative cell damage can lead to permanent loss of neurological function.[2,3] The primary damage results from trauma-associated mechanical tissue disruption; secondary damage is mediated by complex cellular and molecular processes. Treatments that minimize secondary injury and promote survival of spinal cord tissue can preserve the anatomic substrate necessary for functional recovery.[4,5]

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Programmed cell death associated with apoptosis occurs secondary to SCI and results in further neural damage and loss of function. Apoptosis following primary SCI is accompanied by activation of caspase-3, a member of the cysteine protease family,[6] and is regulated by pro- and anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) protein family.[7]

Buyang Huanwu decoction (BYHWD) is a traditional Chinese herbal medicine that has been used in China for hundreds of years to treat SCI,[8] and has been shown to have significant neuroprotective properties.[9] BYHWD has been shown to improve the outcome of SCI in animal models,[10,11] but the mechanisms of action are not fully understood. Recent investigations found that the neuroprotective effects of BYHWD on spinal cord neurons were associated with

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DOI 10.1179/2045772314Y.0000000273

apoptosis[12] and a few studies have linked BYHWD treatment with angiogenesis, but again, the mechanisms are not known. This study investigated the neuroprotective effects of BYHWD and the expression of caspase-3, Bax, and Bcl-2 mRNA and protein after SCI. The rationale for the study is the need for additional data to support the development of novel, effective therapeutic alternatives to treat SCI.

Materials and methods

Animals and drugs

The experimental procedures were performed using adult female Wistar rats weighing 300–350 g. Animals fed with standard animal chow and with free access to water were housed in individual, standard rat cages and maintained in a controlled environment at 22 ± 1°C and a 12-hour light–dark cycle. The Medical Ethics Committee of Chengde Medical University, China gave full approval for this study.

BYHWD is composed of *Radix astragali*, *R. angelicae sinensis*, *R. paeoniae rubra*, *Rhizoma chuanxiong*, *Semen persicae*, *Flos carthami*, and *Lumbricus*. The decoction was made by boiling the mixture three times in distilled water at 100°C for 30 minutes. The drug solution was then vacuum-cooled and dried to form a powder. The *R. astragali* (Fig. 1A), *R. angelicae sinensis* (Fig. 1B), *R. paeoniae rubra* (Fig. 1C), *R. chuanxiong* (Fig. 1D), *S. persicae* (Fig. 1E), *F. carthami* (Fig. 1F), and *Lumbricus* (Fig. 1G) powders were mixed at a ratio of 120:10:10:10:10:10:4.5 and were then dissolved in distilled water at a final concentration of 2 g/ml (equivalent to the dry weight of the raw materials).[13] All materials were provided without charge by the China Resources Sanjiu Medical and Pharmaceutical Co., Ltd China (Fig. 1).

Spinal cord injury

SCI was induced surgically as previously described; aseptic technique and sterile instruments were used.[14] Rats were anesthetized and maintained with isoflurane in O_2 for the procedure.[15] After routine disinfection, a surgical incision was made through the skin, subcutaneous tissue, and the T8–T12 vertebral laminae to expose the spinal canal (Fig. 2A). The rostral and caudal spinous processes were fixed by clamping and SCI was induced using an impactor (Fig. 2B) that was made in house and was similar to the device used in the New York University-Multicenter Animal Spinal Cord Injury Study (NYU-MASCIS). The impactor weighed 10 g and was dropped once from a height of 25 mm, which was assumed to produce severe SCI based on the results of a prior investigation.[16] After injury (Fig. 2C), a total laminectomy of T9 or T10

Figure 1 Powdered ***R. astragali***, ***R. angelicae sinensis***, ***R. paeoniae rubra***, ***R. chuanxiong***, ***S. persicae***, ***F. carthami***, and ***Lumbricus***. All materials were kindly provided by China Resources Sanjiu Medical and Pharmaceutical Co., Ltd. (A) ***R. astragali***, (B) ***R. angelicae sinensis***, (C) ***R. paeoniae rubra***, (D) ***R. chuanxiong***, (E) ***S. persicae***, (F) ***F. carthami***, (G) ***Lumbricus***.

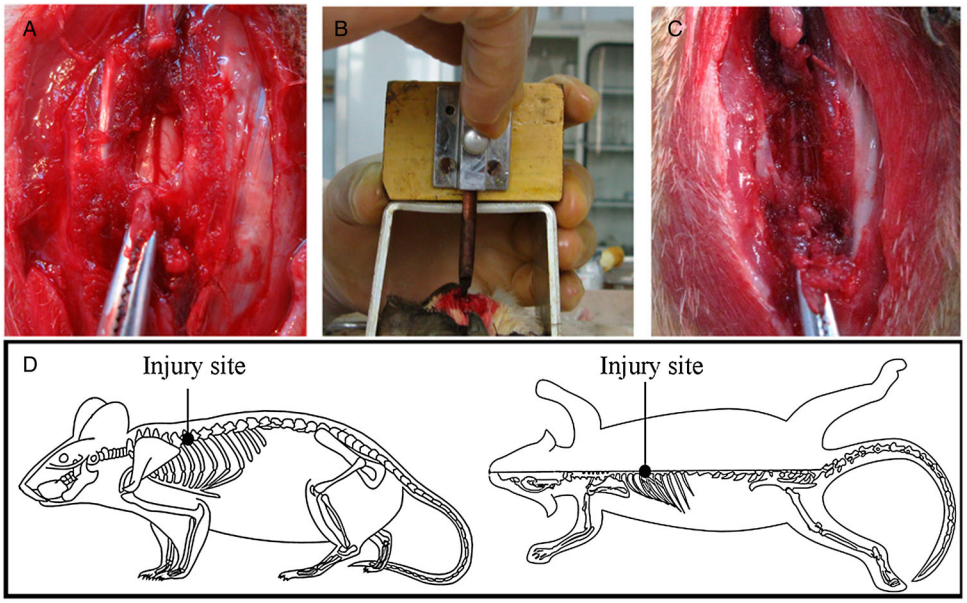

Figure 2 (A) Exposure of the spinal cord at T10–11 by laminectomy. (B) Special impactor equipment made in our laboratory. (C) The spinal cord after injury. (D) The injury site.

was performed (Fig. 2D). Animals subjected to identical surgical procedures, without impaction, served as sham-operated controls. All procedures were approved by the Chengde Medical University Animal Studies Committee. Perioperative care followed MASCIS guidelines as described in previous publications.[17]

Groups and treatments

Forty rats with similar locomotor ability were randomized to four groups of 10 animals each using a table of random numbers. The group treatments included sham surgery, SCI, BYHWD treatment, and methylprednisolone (MP) treatment. The MP group was injected with a high dose of a glucocorticoid steroid (MP) once daily for 4 weeks beginning 2 hours after surgery. BYHWD was administered orally twice daily (4 g/day) for 4 weeks using an orogastric tube starting 2 hours after surgery. The sham-operated and SCI groups were given distilled water following the same regimen as BYHWD.

Neurological assessment

Locomotor function was evaluated 1 day before surgery, 1 day after surgery, and then weekly using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale.[18] For testing, rats were placed in a large open area so that aversion to open spaces stimulated them to move towards the field borders.[19] Hip, knee, and ankle joint movements and trunk, tail, and hind-paw positions were observed and scored. The scores ranged from 0 to 21, with 0 indicating total absence of movement and 21 indicating normal movement. Four independent, blinded examiners observed the hind-limb movements and assessed locomotor function.[20] Rats falling more than two standard deviations above or below the group mean BBB scale score were excluded from the final statistical review. Left and right limb movements were recorded and analyzed separately, but were averaged in the final analysis.

Tissue processing

The animals were sacrificed at 4 weeks. Four rats from each group were anesthetized with chloral hydrate and transcardially perfused with tyrode buffer followed by a tissue fixative solution (4% paraformaldehyde, 15% picric acid, and 0.05% glutaraldehyde in phosphate buffer). The T12–T14 spinal cord was removed, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight, embedded in paraffin, sectioned (5 μm), and stored at room temperature until assayed for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

The six remaining rats in each group were sacrificed without perfusion, and the T12–T14 spinal cord was quickly removed, and divided into two parts on an ice-cold surface. It was kept at –80°C for real-time quantitative polymerase chain reaction (qPCR) and Western blotting.

TUNEL assay and immunohistochemical staining

TUNEL for detection of apoptotic cells was performed using the ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's protocols. Briefly, 5 μm thin tissue sections mounted on silanized slides were

dewaxed, washed with PBS and pretreated with proteinase K (20 mg/ml) (Millipore, USA) for 15 minutes. Sections were then quenched in 3% hydrogen peroxide for 5 minutes, covered with equilibration buffer for 10 seconds, and incubated with TdT enzyme at 37°C for 1 hour in a humidified chamber. After labeling, sections were washed and allowed to bind to anti-digoxigenin conjugate in a humidified chamber for 30 minutes, stained with 3,3′-diaminobenzidine peroxidase substrate for 5 minutes, counter-stained with 0.5% methyl green for 10 minutes, and coverslipped. Slides were examined by light microscopy and five random fields of view in each section were photographed. The percentage of apoptotic cells was scored as the average ratio of TUNEL-positive cells to total cells counted in each field.

qPCR

Total RNA was extracted from tissues using the RNA Easy Mini Kit (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Caspase-3, Bax, and Bcl-2 mRNA were detected by qPCR using a Light Cycler System (Roche Diagnostics K. K., Tokyo, Japan). qPCR amplifications were performed in 25 μl aliquots containing 2.5 μl cDNA, 2.5 μl mixed gene-specific forward and reverse primers (10 mM each), 12.5 μl of 2 × SYBR Premix Ex Taq (Invitrogen, Grand Island, NY, USA) and 7.5 μl double-distilled water. qPCR was carried out with a Miniopticon instrument (Bio-Rad, Hercules, CA, USA). The amplification reaction comprised an initial 30 seconds predenaturation at 95°C, followed by 40 cycles of two-step PCR denaturation at 95°C for 5 seconds, and annealing/extension at 57°C for 30 seconds. Differences in RNA quantity and quality among the samples tested were normalized against values for the β-actin gene, which was used as an internal control. For each analyzed sample, qPCR provides a threshold cycle (C_T) value where a fluorescence signal is detectable. All C_T are dependent on the starting amount of cDNA. Quantification of target gene mRNA was performed using the $2^{-\triangle\triangle CT}$ method. The *X*-fold curves were produced as follows: if the control point ratio was 1, the *X*-fold of that time point was calculated as *X*. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to determine whether there were significant variations in the qPCR data. Sequences of the primers used for the analyses in this study are shown in Table 1.

Table 1 Primer sequences of investigated genes

Gene	Primer sequences
β-Actin	F:5′-ACAGCTTCTTTGCAGCTCCTTC-3′
	R:5′-CCACGATGGAGGGGAATACAG-3′
Bax	F:5′-TTTCATCCAGGATCGAGCAG-3′
	R:5′-CAAAGTAGAAGAGGGCAACCAC-3′
Bcl-2	F:5′-GACGAGAAGTGCTATTGGT-3′
	R:5′-TCAGGCTGGAAGGAGAAGAT-3′
Caspase-3	F:5′-TGCGGCGTTACACGACCTT-3′
	R:5′-CAAAGCCAGTGGCACTCATTCTC-3′

Western blotting analysis

Pieces of spinal cord tissue were minced and homogenized in chilled lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM PMSF, 10 mg/ml leupeptin, 0.1% sodium dodecyl sulfate, and 1 mM NaF) overnight at 4°C. Cell pellets were lysed directly on the culture dishes using the same lysis buffer. The lysates were collected, and centrifuged at 12 000 rpm for 30 minutes; the total protein content was then measured. The supernatant (soluble subcellular fraction) was removed, aliquotted, and stored at −80°C.

Fifty micrograms total protein isolated from each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% gradient Tris/glycine gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA) and incubated with anti-caspase-3, anti-Bax, anti-Bcl-2, and anti-β-actin primary monoclonal antibodies overnight at 4°C after blocking. The blots were washed with 5% powdered milk in PBS/0.1% Tween 20 prior to incubation with horseradish peroxidase-conjugated IgG secondary antibody at room temperature for 1 hour. Western blots were quantified by densitometry and normalized against β-actin. Immunoreactive bands were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) using the ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA) with the Image-Pro plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

Results were expressed as means ± standard deviation. Repeated measurements score data were analyzed using multivariate analysis of variance (ANOVA). Additional statistical analysis included univariate ANOVA of within-group data. Bonferroni *post hoc* analysis was conducted following significant ANOVA results. The comparisons of interest were the SCI group versus the sham-operated control group, and the drug-treated groups versus the SCI group. All analyses were performed using the SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) with the level of significance set at $P < 0.05$.

Results

Effect of BYHWD on SCI alteration of BBB scores

Walking was mildly abnormal in sham-operated rats after anesthesia, followed by recovery after 2 days. The BBB scores of rats in the SCI model group were significantly different than scores of rats in the sham control group at 1, 7, 14, 21, and 28 days after injury. The BBB scores of rats in both the BYHWD and MP groups were significantly different than scores of rats in the SCI group (Fig. 3).

Effect of BYHWD on SCI alteration in TUNEL

To determine whether BYHWD inhibited the apoptosis of spinal nerve cells, we examined the DNA fragmentation in these tissues using the TUNEL assay. Representative tissue sections stained with TUNEL are shown in Fig. 4. Significantly fewer apoptotic cells were observed in the sham group (1.47%, Fig. 4A) than in the SCI group (42.65%, Fig. 4B). The number of apoptotic cells in the BYHWD (11.76%, Fig. 4C) and MP (10.29%, Figure 4D) groups were significantly lower than in the SCI group (Fig. 4E).

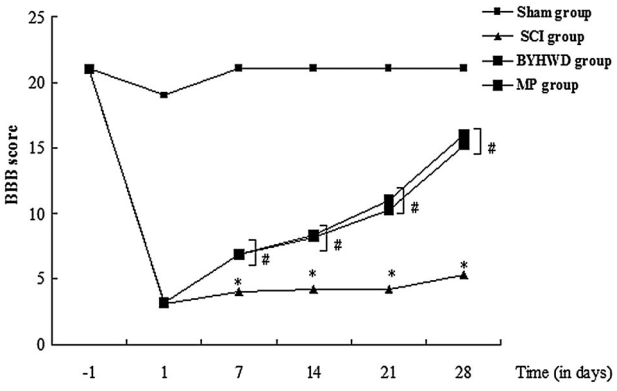

Figure 3 Effect of BYHWD on SCI alteration of BBB scores. At 1, 7, 14, 21, and 28 days after surgery, the BBB score of rats in the SCI model group were significantly different than scores of rats in the sham control group. The BBB scores of rats in the BYHWD and MP groups were significantly different than those of rats in the SCI group. $n = 10$ in each group; *P < 0.05 compared with the sham group; #P < 0.05 compared with the SCI model group.

Effect of BYHWD on expression of caspase-3, Bax, and Bcl-2 mRNA

The qPCR results revealed that BYHWD decreased mRNA expression of caspase-3 and Bax, but increased the mRNA expression of Bcl-2 (Fig. 5).

Effect of BYHWD on expression of caspase-3, Bax, and Bcl-2 protein

Western blot analysis found that BYHWD decreased expression of caspase-3 and Bax protein, but increased the protein expression of Bcl-2 (Fig. 6).

Discussion

The consequences of SCI caused by trauma are devastating.[21] The victims who suffer the most are those with quadriplegia associated with autonomic dysfunction. Long-term survival is frequent in most patients with SCI, and as they are predominantly relatively young, working-age people who require prolonged rehabilitation, the personal and social impacts are extremely deleterious.[22]

SCI can be divided into primary and secondary SCI.[23] In addition to the damage at the site of the SCI, secondary pathological changes occur in the following order: edema, ischemia, calcium overload, lipid

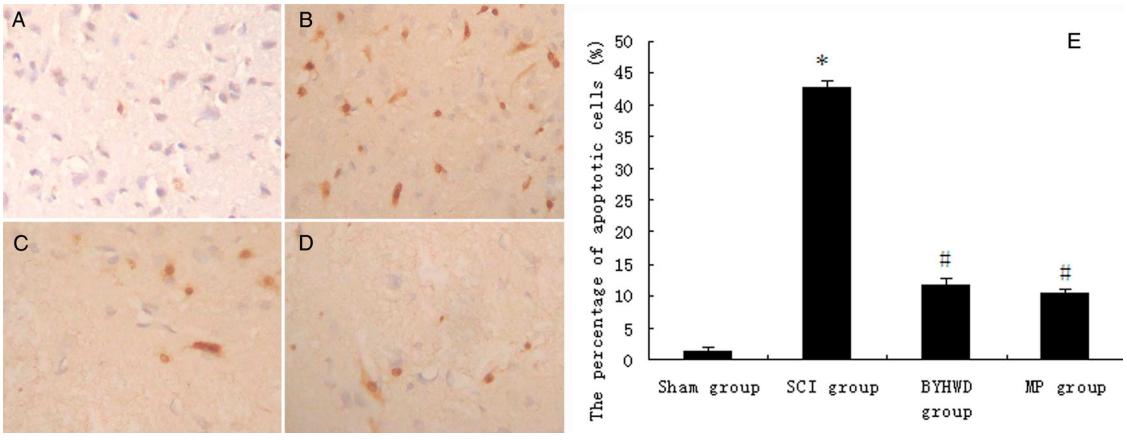

Figure 4 Effect of BYHWD on SCI alteration of TUNEL assay. (A) Apoptotic cells in the sham group. (B) Apoptotic cells in the SCI group. (C) Apoptotic cells in the BYHWD group. (D) Apoptotic cells in the MP group. (E) Quantification of apoptotic cells. Significantly more apoptotic cells were observed in the SCI group (42.65%) than in the sham group (1.47%). There were significantly fewer apoptotic cells in the BYHWD (11.76%) and MP groups (10.29%) than in the SCI group. $n = 4$ in each group; *P < 0.05 compared with the sham group; #P < 0.05 compared with the SCI model group.

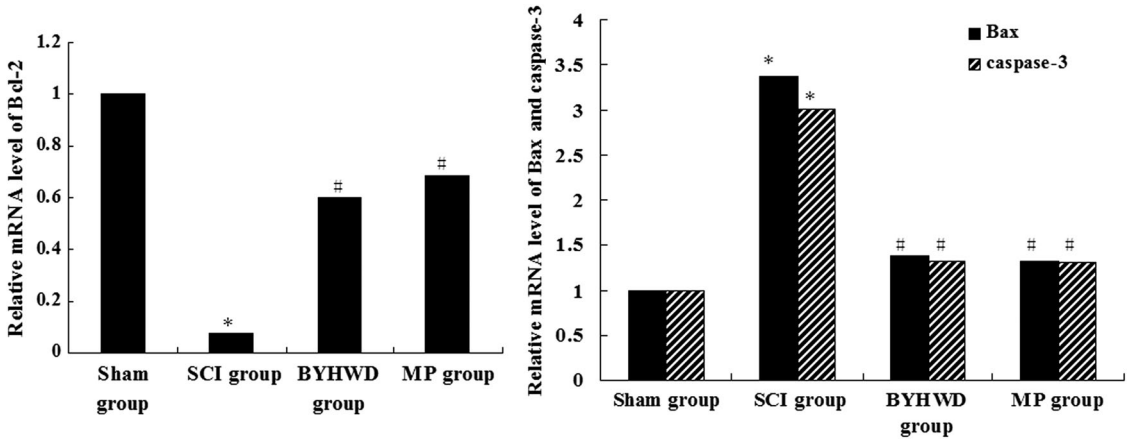

Figure 5 Effect of BYHWD on expression of caspase-3, Bax, and Bcl-2 mRNA by qPCR. BYHWD decreased caspase-3 and Bax mRNA expression but increased the expression of Bcl-2 mRNA. $n = 6$ in each group; *P < 0.05 compared with the sham group; #P < 0.05 compared with the SCI model group.

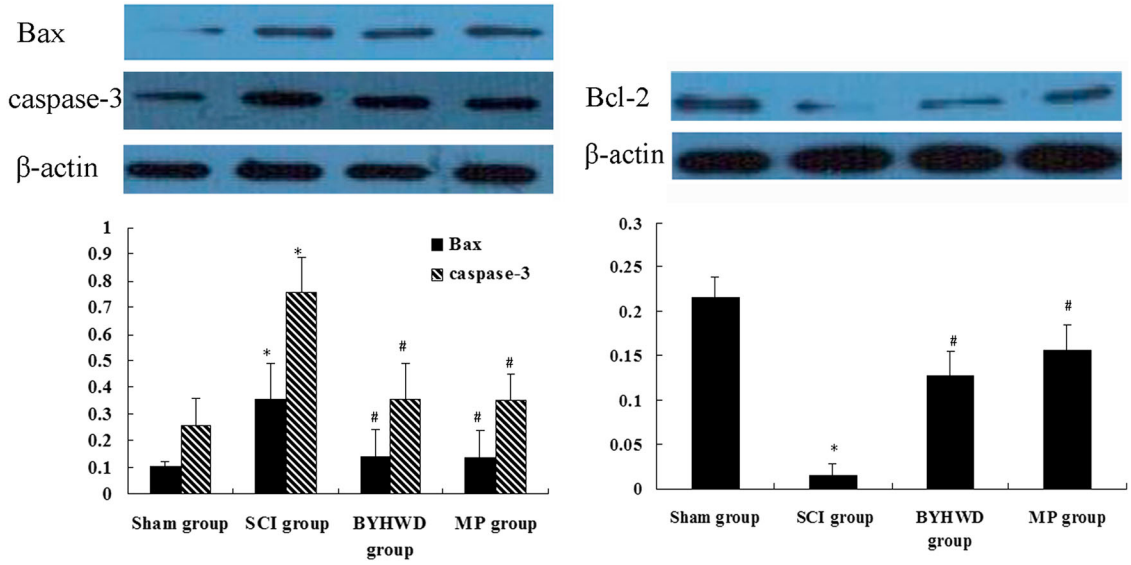

Figure 6 Effect of BYHWD on caspase-3, Bax, and Bcl-2 protein expression by Western blotting. BYHWD decreased expression of caspase-3 and Bax protein but increased the expression of Bcl-2 protein. $n = 6$ in each group; *P < 0.05 compared with the sham group; #P < 0.05 compared with the SCI model group.

peroxidation, microcirculation obstruction, and apoptosis.[24] One of the most validated secondary injury mechanisms in acute SCI is post-trauma increase in apoptosis of nerve cells. This apoptosis response is a key target of successful therapy.[25,26]

The impactor tool employed in this study was made at our laboratory, and has been used there for years.[27] It produces a validated and reproducible contusion injury in rat models.

BYHWD is a traditional Chinese herbal medicine that is used to treat SCI. Its neuroprotective effects have been demonstrated in cerebral ischemia-reperfusion injury,[28,29] and may involve a number of mechanisms, including increased nerve proliferation, improved nerve regeneration, and reduced inflammatory cytokine and neuronal apoptosis.[30] These mechanisms are strongly associated with the pathophysiology and clinical outcome of the acute and subacute phases of cerebral ischemia.[8]

MP is used to treat a variety of neurological disorders involving white matter injury, including multiple sclerosis, acute disseminated encephalomyelitis, and SCI.[31,32] It was used here as a positive control; its marginal effectiveness and a poor side-effect profile have raised concerns.

The BBB scale score was used to monitor functional recovery and locomotor ability in this SCI study. The scale range (0–21) represents sequential recovery

stages and was used to categorize combinations of rat joint and hind-limb movement, stepping, fore-limb and hind-limb coordination, trunk position and stability, paw placement, and tail position. To evaluate bladder contraction, urodynamics were studied by serial cystometrograms. The study results showed that BYHWD treatment significantly improved the recovery of locomotor function in rats with induced SCI versus an SCI group without BYHWD treatment. Treatment with MP, which served as a positive control, had similar effects.

TUNEL is frequently used to detect DNA fragmentation that results from apoptotic signaling cascades. Significantly fewer TUNEL-positive neurons were seen in spinal cords from rats treated with BYHWD than in untreated SCI rats, a result that was associated with partially restored function of neurons. The data suggested that both BYHWD and MP may promote the recovery from injury lesions.

The qPCR and Western blot results showed that BYHWD prevented apoptosis of neurons following SCI. This neuroprotective effect might result from decreased expression of caspase-3 and Bax and increased Bcl-2 expression in response to BYHWD.

Conclusion

Although exclusion of rats with BBB scores more than two standard deviations from the group mean might add selection bias, and substantial heterogeneity existed, sensitivity analyses found that the analysis was consistent and robust. In summary, the data showed that BYHWD improved the recovery of nerve function by reducing apoptotic cell death in this rat model of SCI. The neuroprotective effects of BYHWD treatment may be mediated in part by antiapoptosis effects following injury. In addition, BYHWD may prevent apoptosis of neurons following SCI. The possible mechanism of this antiapoptosis activity may involve decreased expression of caspase-3 and Bax, and increased Bcl-2 expression.

Acknowledgments

This study was supported by the traditional Chinese medicine of Hebei province of China, contract grant number 2012049.

Disclaimer statements

Contributors All the authors have played an important role.

Funding Funding The traditional Chinese medicine of Hebei province of China, NO.2012049.

Conflicts of interest None.

Ethics approval The Medical Ethics Committee of Chengde Medical University, China gave full support for this study.

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