Research Article

Total flavonoids of hawthorn leaves promote motor function recovery via inhibition of apoptosis after spinal cord injury

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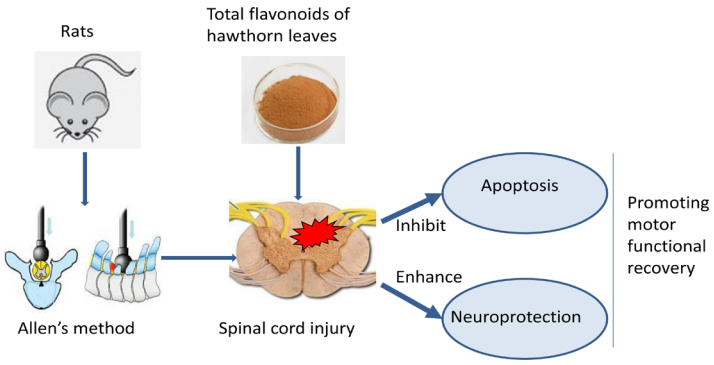
Graphical Abstract *Total flavonoids of hawthorn leaves can reduce apoptosis and exert neuroprotective effects to promote the recovery of the motor function of rats with spinal cord injury*



Abstract

Flavonoids have been reported to have therapeutic potential for spinal cord injury. Hawthorn leaves have abundant content and species of total flavonoids, and studies of the effects of the total flavonoids of hawthorn leaves on spinal cord injury have not been published in or outside China. Therefore, Sprague-Dawley rats were used to establish a spinal cord injury model by Allen's method. Rats were intraperitoneally injected with 0.2 mL of different concentrations of total flavonoids of hawthorn leaves (5, 10, and 20 mg/kg) after spinal cord injury. Injections were administered once every 6 hours, three times a day, for 14 days. After treatment with various concentrations of total flavonoids of hawthorn leaves, the Basso, Beattie, and Bresnahan scores and histological staining indicated decreases in the lesion cavity and number of apoptotic cells of the injured spinal cord tissue; the morphological arrangement of the myelin sheath and nerve cells tended to be regular; and the Nissl bodies in neurons increased. The Basso, Beattie, and Bresnahan scores of treated spinal cord injury rats were increased. Western blot assays showed that the expression levels of pro-apoptotic Bax and cleaved caspase-3 were decreased, but the expression level of the anti-apoptotic Bcl-2 protein was increased. The improvement of the above physiological indicators showed a dose-dependent relationship with the concentration of total flavonoids of hawthorn leaves. The above findings confirm that total flavonoids of hawthorn leaves can reduce apoptosis and exert neuroprotective effects to promote the recovery of the motor function of rats with spinal cord injury. This study was approved by the Ethics Committee of the Guangxi Medical University of China (approval No. 201810042) in October 2018.

Key Words: apoptosis; Bax protein; Bcl-2 protein; cleaved caspase-3; inflammation; motor function recovery; neuroprotection; Nissl bodies; spinal cord injury; total flavonoids of hawthorn leaves

Chinese Library Classification No. R453; R363; R364

Introduction

Spinal cord injury (SCI) is a serious condition that lacks effective therapeutics and exhibits poor healing. Currently, the main treatment method for SCI is surgery combined with methylprednisolone (Ahuja et al., 2017; Liu et al., 2019). Although this method has shown some advantages, it has led to serious trauma and given rise to many side effects. Therefore, identification of a gentle and effective treatment is particularly important. Related studies have revealed that flavonoids have therapeutic potential in SCI (Zhang et al., 2017), and flavonoids are widely distributed in many plants. Although some scholars have explored the effects of flavonoid

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extracts of different plants such as *Astragalus membranaceus* and *Salvia miltiorrhiza* on SCI (Zhang et al., 2018, 2019), the question of which flavonoids have the best effects on SCI still remains unresolved. Therefore, it is important to explore the effects of new flavonoids on SCI.

SCI is mainly caused by a severe external mechanical impact on the spinal cord that destroys the original intact tissue structure and triggers a series of cellular and molecular reactions, such as inflammation, autophagy and apoptosis (Takenaga et al., 2006; Li et al., 2017). SCI is divided into primary SCI and secondary SCI based on the mechanism of injury (Piltti et al., 2017; Zhou et al., 2017). Primary SCI refers to the destruction of the blood-brain barrier, hemorrhage, edema, axonal destruction, and cell membrane decomposition caused by an external force (Oliveri et al., 2014; Yu et al., 2020). Secondary SCI induces a series of biochemical changes, such as glial activation and inflammatory cytokine expression, in response to the primary injury, which in turn aggravates the degree of primary SCI (Maggio et al., 2012; Zhao et al., 2016). SCI is mainly characterized by partial or complete loss of motor and sensory functions (Park et al., 2014; Wang et al., 2018; Tsai et al., 2019). Related studies have shown that apoptosis is a major cause of motor and sensory function deficits (Ola et al., 2011; Rahman et al., 2012), and inhibition of apoptosis can improve these defects (Kwon et al., 2015). Apoptosis is mainly characterized by nuclear pyknosis, DNA fragmentation, excitation of caspase-3, and fluctuation in the expression of Bcl-2 family proteins such as Bax and Bcl-2 (Penkowa et al., 2006; Zhu et al., 2018).

Total flavonoids of hawthorn leaves (TFHL) is a generic term for a series of flavonoids extracted from hawthorn leaves. TFHL has anti-inflammatory, antioxidant, and anti-ischemic activities (Wei et al., 2017; Wu et al., 2018). Studies have found that TFHL can alleviate inflammation, inhibit apoptosis, and affect the expression of caspase-3, Bcl-2 Bax, and Bcl-2 proteins (Dong et al., 2017; Alirezalu et al., 2018), but most of these claims are based on myocardial injury, kidney injury, and other models. However, the function of TFHL in SCI remains unknown.

Therefore, using SCI model rats, this study investigated whether TFHL can alleviate apoptosis and exert neuroprotective effects to enhance the recovery of motor function in rats with SCI.

Materials and Methods

Experimental animals

Thirty specific-pathogen-free Sprague-Dawley rats aged 4–6 weeks and weighing 200–250 g (half females and half males) were purchased from the Animal Experimental Centre of Guangxi Medical University (license No. SCXK (Gui) 2014-0005). The animals were raised under a 12-hour light/dark cycle at 20–25°C with a relative air humidity of 50–60%, were fed a standard diet, and had free access to water. All animal experimental procedures were approved by the Ethics Committee of the Guangxi Medical University of China (approval No. 201810042) in October 2018. The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

SCI model establishment and drug administration

TFHL was purchased from Shanxi Kanglisheng Pharmaceutical Co., Ltd., China (product batch No. 15118240). TFHL is a light yellow powder, and its main ingredients include (1) quercetin-3-O-(2, 6-di-α-L-rhamnopyranosyl)-β-D-galactopyranoside, (2) vitexin, (3) quercitrin, (4) isoquercitrin, (5) hyperoside, (6) vitexin-4′′-O-glucoside, (7) epicatechin, (8) vitexin-2′′-O-rhamnoside, and (9) vitexin-6′′-O-acetyl (Ma et al., 2010; Wen et al., 2017). According to the body weight of the rats, TFHL was weighed and then dissolved in 1 mL of physiological saline at concentrations of 5, 10, and 20 mg/kg. Bacteria were filtered using a 0.22 μm filter (Pall, Shanghai, China), and the solutions were stored for future use.

The thirty rats were equally and randomly divided into a sham operation group, SCI group, and SCI + 5, 10, or 20 mg/kg TFHL groups. The SCI model was established using Allen's method (Allen, 1911). After intraperitoneal anesthesia was administered with 0.7 mL/100 g of 5% chloral hydrate (Maclin, Suffolk, UK), the rats were fixed in the prone position. The skin of the surgical area was routinely sterilized with iodophor and perforated. The skin incision was 3 cm, and the subcutaneous fascia and paravertebral muscles were separated. The spinous process and interspinous process ligament were cut. The T9–11 spinous processes were fully exposed. The ligamentum flavum was gently redirected between the laminae, and the T10 lamina was clamped with forceps to expose the dura mater. The dural sac was struck with the Allen device (10 g Kirschner wire, Jiangzhou Medical Equipment Co., Ltd., Jiangsu, China), which fell freely from a height of 3 cm, causing impact damage to the T10 segment of the spinal cord. The sham operation group underwent the same procedure as the SCI group, except that the Allen device was not used to damage the T10 segment of the spinal cord. After injury, the wound was washed with warm saline, and the muscle, fascia, and skin were sutured in sequence. The surface of the injured spinal cord quickly oozed dark red blood. After the operation, the rats were housed in a single cage, and 30,000 units of penicillin was subcutaneously injected once a day. Urinary bladder pressure was applied twice a day for 3 days to encourage voiding. After 3 days, the hind limbs of the rats had no conditioned reflex under stimulation. The vital signs of rats were stable, and no deaths occurred, which indicated that the SCI model was successfully established. The three treatment groups of rats were intraperitoneally injected with 0.2 mL of different concentrations of TFHL (5, 10, and 20 mg/kg) after surgery. Injections were administered once every 6 hours, three times a day, for 14 days. For the sham operation and SCI groups, the same volume of physiological saline was intraperitoneally injected, and the number and timing of administrations were the same as those of the treatment groups.

After establishing SCI models and performing TFHL treatment, injured spinal cord tissue was extracted for testing. Each group was randomly divided into two sub-groups with three rats in each sub-group. Samples were extracted from one sub-group for hematoxylin-eosin staining and electron microscopy. The other sub-group was used for Nissl staining and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining. The remaining tissues of each group were extracted for western blot assays.

Motor function test

The first day after the model was successfully established, limb movement corresponding to the injured section of the spinal cord in the rats was observed on a flat surface. The hind limb motor function of the five groups of rats was evaluated by the Basso, Beattie, and Bresnahan (BBB) scoring method (Basso et al., 1995, 1996). The scoring criteria were divided into three parts: Part 1 (0–7 points), assessment of hind limb joint activity in the SCI segment of rats; Part 2 (8–13 points), assessment of SCI in rats according to hind limb gait and coordination function; and Part 3 (14–21 points), assessment of fine movement of the paw during exercise in rats. The movement of the rats was observed, and the scores were recorded by three observers who were blinded to the treatments. Higher scores indicated better motor function of the limb movement of rats. The average of three scores was recorded as the final score of the rat motor function test.

Hematoxylin-eosin staining

After 14 days of continuous treatment with TFHL, T10 segments of the spinal cord tissues of the five groups of rats were separately extracted and fixed in liquid paraffin (Sinopharm Group; 69019361, Shanghai, China) to prepare paraffin sections. The prepared paraffin sections were dewaxed in xylene (Sinopharm Group; 10023418) and hydrated with an ethanol concentration gradient. After three washes with phosphate-buffered saline (PBS), 100 µL of pre-formulated hematoxylin staining solution was added to each tissue section, which was then stained for 10 minutes. The excess hematoxylin staining solution was washed with distilled water, and then the sections were differentiated using 1% hydrochloric acid in ethanol. Afterwards, the tissue sections were washed with ultra-pure water, stained with Yihong dye solution for 3 minutes, dehydrated with different concentrations of ethanol, soaked in xylene, air-dried, and finally sealed with a neutral resin. The tissue sections were observed under an upright fluorescence microscope (Olympus BX53, Tokyo, Japan). The proportions of lesion cavity areas in different groups were analyzed using ImageJ 5.0 software (Rawak Software Inc., Stuttgart, Germany). The above analysis was completed by three researchers who were blinded to the treatments.

Transmission electron microscopy

After 14 days of continuous treatment with TFHL, the T10 segments of the spinal cord in the five groups of rats were quickly removed, rinsed with saline, and fixed with 2.5% glutaraldehyde buffer (Maclin; G849973, Suffolk, UK) at 4°C for more than 2 hours. The spinal cord tissues were washed three times with PBS, 45 minutes each, and fixed with 1% osmium tetroxide buffer (Maclin; P816056) at 4°C for 2 hours. After three washes with PBS, the spinal tissues were subjected to dehydration with a gradient consisting of 70%, 80%, and 90% ethanol (Sinopharm Group; 10009218) and 90% and 100% 1:1 ethanol and acetone (90% ethanol: 90% acetone) (Aladdin; S104174, Shanghai, China). The tissues were infiltrated with propylene oxide and an embedding agent, embedded with epoxy resin (Sigma-Aldrich, St. Louis, MO, USA; 430234), and then sliced into ultrathin sections. The sections were stained with saturated uranium acetate and lead citrate (ALFA, Haverhill, MA, USA; A04A10701) for 30 minutes and 8 minutes, respectively, and finally placed under a scanning transmission electron microscope (HITACHI H-7650, Tokyo, Japan) for observation.

Nissl staining

After 14 days of continuous treatment with TFHL, the tissue paraffin sections were dewaxed and hydrated. After washing with distilled water, the sections were stained with 0.25% toluidine blue (Sinopharm Group; 71041284) at 60°C for 3 hours. The remaining dye solution was quickly washed with ultra-pure water, and then the sections were washed with 95% ethanol, dehydrated with absolute ethanol, made transparent with xylene (Sinopharm Group; 10023418), and then mounted with neutral gum (Sinopharm Group; 10004160). The sections were observed, and images were captured under an upright fluorescence microscope (Olympus BX53, Tokyo, Japan). The number of Nissl bodies/mm^2 was analyzed by ImageJ 5.0 software (Rawak Software Inc., Stuttgart, Germany). The above analysis was completed by three researchers who were blinded to the treatments.

TUNEL staining

To detect cell apoptosis in injured spinal cord tissue after 14 days of continuous treatment with TFHL, pre-treated tissue sections were examined using a TUNEL Apoptosis Detection Kit (Jiamay; TUN11684817, Beijing, China) according to the method described in the instruction manual. The tissue sections were pretreated with 20 mg/mL proteinase-k for 15 minutes at normal temperature, and the prepared TUNEL reaction mixture solution was added and incubated at 37°C for 1 hour. After the sections were air-dried, 50 µL of converter-POD was added to react with the sections in a 37°C incubator for 30 minutes, and then 50 µL of 3,3′-diaminobenzidine substrate was added and reacted at 15°C for 10 minutes. After TUNEL staining, the nuclei were stained with 4′,6-diamidino-2-phenylindole (Beyotime; C1002, Shanghai, China). The prepared TUNEL-stained sections were observed under an upright fluorescence microscope (Olympus BX53, Tokyo, Japan). The percentage of TUNEL-positive cells among all cells in the spinal cord tissues of the different groups was recorded. The above positive cell counts were completed by three researchers who were blinded to the treatments.

Western blot assay

After 14 days of continuous treatment with TFHL, the spinal cord tissues of rats were disrupted. A pre-prepared lysate (PIRA lysate + PMSF protease inhibitor; Cell Signaling Technology, Danvers, MA, USA) was added, and the supernatant was extracted by centrifugation at 10,000 × *g* for 15 minutes at 4°C. The protein sample concentration was determined using a BCA Protein Assay Kit (Beyotime) prior to protein loading. After 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, the proteins on the gel were transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked at room temperature for 1 hour with Tris buffer containing tween 20 and nonfat dry milk. Then, the membrane was incubated with the primary antibody (rabbit Bcl-2 antibody, 2870s, 1:1000; rabbit Bax antibody, 14796s, 1:1000; or rabbit cleaved caspase-3 antibody, 9664s, 1:1000; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Mouse anti-β-actin (1:1000, Cell Signaling Technology, Danvers, MA, USA) was used as an internal control. The membrane was incubated with the secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated IgG, 1:5000; Jackson Immunochemicals, West Grove, PA, USA) at room temperature for 1.5 hours. The membranes were observed on a GE Amersham Imager 600 (General Electric, Boston, MA, USA). The gray value ratio of protein bands was quantified using ImageJ 5.0 software (Rawak Software Inc., Stuttgart, Germany).

Statistical analysis

The experimental data are expressed as the mean ± standard deviation (SD) and were analyzed by SPSS 24.0 statistical software (IBM, Armonk, NY, USA). One-way analysis of variance was used to compare differences among the groups, and the least-significant difference test was used to compare differences between two groups. A value of $P < 0.05$ was considered statistically significant.

Results

TFHL improves motor function recovery of SCI rats

The sham operation group had a BBB score of 21, indicating good motor function. The SCI group had a significantly lower BBB score and significantly impaired motor function ($P < 0.05$). Compared with the SCI group, BBB scores was significantly increased in the SCI + 5, 10, and 20 mg/kg TFHL groups, and the scores increased in a dose-dependent manner with increasing TFHL concentration. The effect of TFHL was most obvious on day 14 ($P < 0.05$; **Figure 1**).

TFHL alleviates spinal cord tissue damage in SCI rats

The results of hematoxylin-eosin staining showed that at 40× magnification, the spinal cord tissue structure of the sham operation group was intact, and the white matter and grey matter of the spinal cord could be clearly distinguished. No inflammatory cell infiltration was observed at 100× magnification. The SCI group revealed incomplete spinal cord

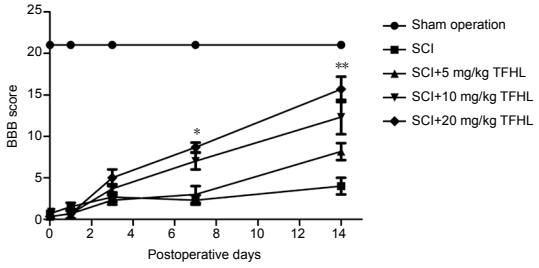

Figure 1 | **Effect of TFHL on motor function recovery of SCI rats.**
BBB scores of different groups were recorded at 0, 1, 3, 7, and 14 days after SCI. All rats in the sham operation group exhibited a BBB score of 21 for the entire measurement period. The SCI group had the lowest BBB score. After 14 days of continuous treatment with TFHL (5, 10, and 20 mg/kg), the scores of the SCI + TFHL groups gradually increased with increasing drug concentration. However, the BBB scores were considered statistically significant among groups at 7 and especially 14 days (*P < 0.05, **P < 0.001). Data are expressed as the mean ± SD (n = 6; one-way analysis of variance followed by a least-significant difference *post hoc* test). BBB: Basso, Beattie, and Bresnahan; SCI: spinal cord injury; TFHL: total flavonoids of hawthorn leaves.

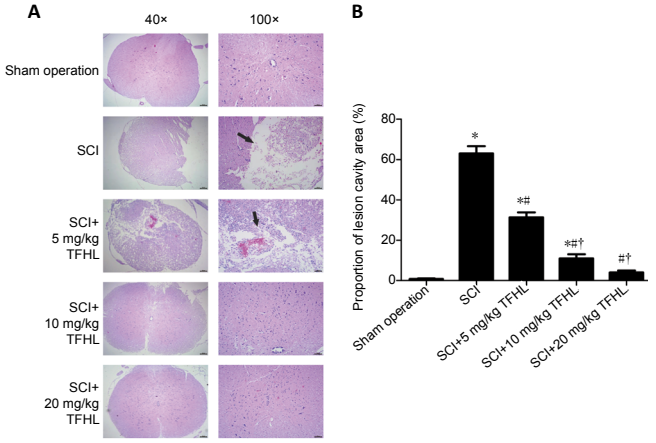

Figure 2 | **Effect of TFHL on histomorphological changes of the injured spinal cord.**
(A) Assessment of injury in spinal cord sections of different groups using hematoxylin-eosin staining (n = 3 per group, cross-section of the T10 segment of the spinal cord, scale bars: 100 μm). Inflammatory cells and the lesion cavity are clearly visible in each group. The arrows indicate severely injured cavities caused by inflammation. It is worth noting that because of injury or technical reasons, the spinal cord morphology data of the spinal cord injury (SCI) group are incomplete, but this did not affect the observation. (B) The proportion of the lesion cavity area of the total area was determined. The SCI group had the largest proportion of lesion cavity. After 14 days of continuous treatment with TFHL (5, 10, and 20 mg/kg), the proportions of lesion cavity areas of the SCI + TFHL groups decreased with increasing drug concentration. *P < 0.05, *vs*. sham operation group; #P < 0.05, *vs*. SCI group; †P < 0.05, *vs*. SCI + 5 mg/kg TFHL group. Data are expressed as the mean ± SD (one-way analysis of variance followed by a least-significant difference *post hoc* test). SCI: Spinal cord injury; TFHL: total flavonoids of hawthorn leaves.

tissue structure at 40× magnification, with a lesion cavity and infiltration of numerous inflammatory cells at 100× magnification. Compared with the SCI group, the spinal cord tissue structure gradually became more intact; the white matter and grey matter structures were not significantly disordered; and the inflammatory cell infiltration was reduced with increasing drug concentration in the SCI + TFHL groups at 100× magnification (**Figure 2A**). Compared with the sham operation group, the lesion cavity proportion was obviously larger in the SCI group (P < 0.05). Compared with the SCI group, the lesion cavity proportion decreased in the SCI + 5, 10, and 20 mg/kg TFHL groups (P < 0.05), and the decrease was dependent on the concentration of TFHL (P < 0.05; **Figure 2B**).

Changes in the ultrastructure of injured spinal cord tissues observed by electron microscopy

In the sham operation group, the shape and structure of the myelin sheath were regular; the arrangement was neat and compact; the nucleus was large and round; and the mitochondria were full. In the SCI group, the shape and arrangement of the myelin sheath were irregular and disordered; the slab structure was loosely arranged, broken, and twisted; the mitochondria were swollen; and the nucleus was irregular. In the SCI + 5, 10, and 20 mg/kg TFHL groups, the situation was improved compared with the SCI group; the shape and structure of the myelin sheath were more regular, orderly, and complete; the lamellar structure was relatively compact; and mitochondrial swelling was reduced. The effect was especially obvious in the SCI + 20 mg/kg TFHL group (**Figure 3**).

TFHL improves the functional status of neuronal cells of SCI rats

The Nissl staining results showed that neuronal cells in the sham operation group had an intact structure, regular morphology, obvious nuclei, and abundant Nissl bodies in the cytoplasm. The SCI group exhibited edema of neuronal cells, unclear structure, and atrophied or smaller Nissl bodies in the cytoplasm. In the SCI + TFHL groups, the morphology of neuronal cells was improved, cell edema was reduced, and the numbers of Nissl bodies were increased (**Figure 4A**). Compared with the sham operation group, the number of Nissl bodies was significantly lower in the SCI group (P < 0.05). Compared with the SCI group, the number of Nissl bodies gradually increased in the SCI + 5, 10, and 20 mg/kg TFHL groups (P < 0.05), and this increase was dependent on the concentration of TFHL (P < 0.05; **Figure 4B**).

TFHL inhibits cell apoptosis in injured rat spinal cord tissue

The TUNEL staining results showed that compared with the sham operation group, the number of TUNEL-positive cells was significantly increased in the SCI group. Compared with the SCI group, after 14 days of continuous treatment with TFHL, the number of TUNEL-positive cells was decreased in the SCI + 5, 10, and 20 mg/kg TFHL groups (**Figure 5A**). The percentage of TUNEL-positive cells was significantly higher in the SCI group than that in the sham operation group (P < 0.05). Compared with the SCI group, the percentage of TUNEL-positive cells among the total cells was decreased in the SCI + THFL groups (P < 0.05), and this percentage was dependent on the concentration of TFHL (P < 0.05; **Figure 5B**).

TFHL regulates the expression of proteins that regulate apoptosis in SCI rats

Western blot assays were used to detect the expression levels of Bcl-2, Bax, and cleaved caspase-3 in the spinal cord of injured rats in the different groups after 14 days of continuous TFHL treatment. Western blot assay results showed that compared with the sham operation group, expression levels of Bax and cleaved caspase-3 were significantly increased and the Bcl-2 protein expression level was decreased in the SCI group (P < 0.05). Compared with the SCI group, expression levels of Bax and cleaved caspase-3 were decreased, and the Bcl-2 protein expression level was increased in the SCI + 5, 10, and 20 mg/kg THFL groups (P < 0.05). As the TFHL dose increased, the expression levels of Bax and cleaved caspase-3 decreased, while the Bcl-2 protein expression level increased (P < 0.05; **Figure 6**).

Discussion

SCI often causes serious psychological and economic burdens in patients and their families. Because of the intractability and complexity of the pathological mechanisms involved, SCI has also been a difficult area of medical research (Jalan et al., 2017). Flavonoids have been reported to have therapeutic potential for SCI (Yu et al., 2019; Zhang et al., 2019), and TFHL has a rich variety and high content of flavonoids.

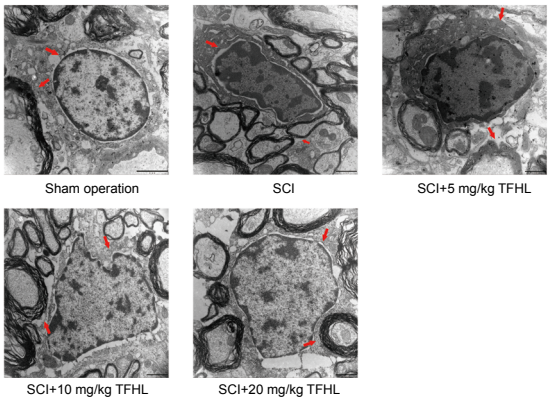

Figure 3 | **Effect of TFHL on ultrastructural changes of the injured spinal cord.**
The changes in ultrastructure of spinal cord tissues were observed by transmission electron microscopy in each group (n = 3; cross-section of the T10 segment of the spinal cord; scale bars: 2 μm for the sham operation group and 1 μm for other four groups). In the sham operation group, the shape and structure of the myelin sheath were regular, the nerve cell nucleus was large and round, and the mitochondria were full. In the spinal cord injury (SCI) group, the shape and arrangement of the myelin sheath were irregular and disordered; the slab structure was loosely arranged, broken, and twisted; the mitochondria were swollen; and the nerve cell nucleus was irregular. In the SCI + TFHL groups, the conditions improved with drug concentration. In particular, the SCI + 20 mg/kg TFHL group demonstrated an obvious effect. Arrows indicate the myelin sheath and nerve cell nucleus of spinal cord tissue. SCI: Spinal cord injury; TFHL: total flavonoids of hawthorn leaves.

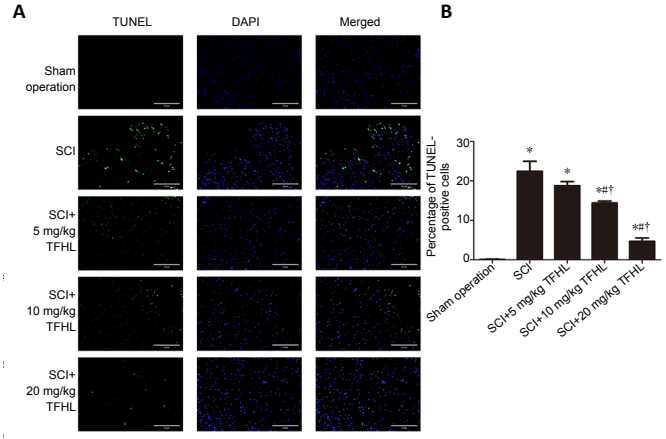

Figure 5 | **Effect of TFHL on cell apoptosis in the injured spinal cord.**
(A) Apoptosis of cells was observed by terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining in each group (n = 3; cross-section of the T10 segment of the spinal cord; scale bars: 200 μm). Apoptotic cells were stained by the TUNEL method, and nuclei were stained with 4′,6-diamidino-2-phenylindole. Green and blue fluorescence represent apoptotic cells and normal cells, respectively. (B) The spinal cord injury (SCI) group exhibited the largest percentage of TUNEL-positive cells. After 14 days of continuous treatment with TFHL (5, 10, and 20 mg/kg), the percentage of TUNEL-positive cells of the SCI + TFHL groups decreased with increasing drug concentration. *P < 0.05, vs. sham operation group; #P < 0.05, vs. SCI group; †P < 0.05, vs. SCI + 5 mg/kg TFHL group. Data are expressed as the mean ± SD (one-way analysis of variance followed by a least-significant difference $post\ hoc$ test). DAPI: 4′,6-Diamidino-2-phenylindole; SCI: spinal cord injury; TFHL: total flavonoids of hawthorn leaves; TUNEL: terminal-deoxynucleotidyl transferase mediated nick end labeling.

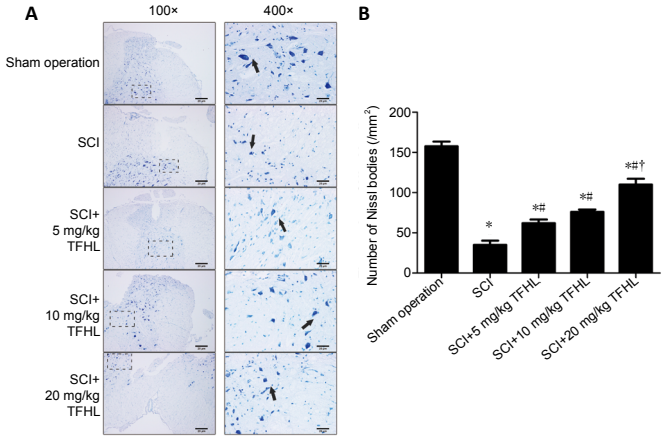

Figure 4 | **Effect of TFHL on the number of Nissl bodies in neurons in the injured spinal cord.**
(A) The number of Nissl bodies in neurons was observed by Nissl staining in each group (n = 3; cross-section of the T10 segment of the spinal cord; scale bars: 200 μm). Blue staining represents a Nissl body, as shown by the arrows. The spinal cord morphology of each group was observed. The Nissl bodies, nucleus, and morphology of nerve cells were clearly visible in each group. (B) The number of Nissl bodies/mm^2 was determined. The spinal cord injury (SCI) group had the lowest number of Nissl bodies/mm^2. After 14 days of continuous treatment with 5, 10, and 20 mg/kg TFHL, the number of Nissl bodies/mm^2 in the TFHL groups increased with increasing drug concentration. *P < 0.05, vs. sham operation group; #P < 0.05, vs. SCI group; †P < 0.05, vs. SCI + 5 mg/kg TFHL group. Data are expressed as the mean ± SD (one-way analysis of variance followed by a least-significant difference $post\ hoc$ test). SCI: Spinal cord injury; TFHL: total flavonoids of hawthorn leaves.

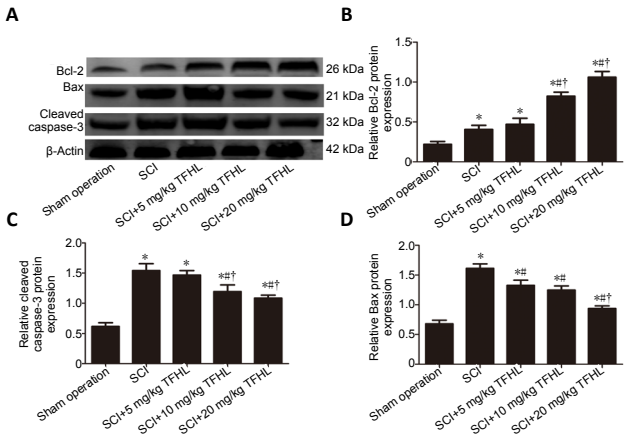

Figure 6 | **Effect of TFHL on expression levels of Bcl-2, Bax, and cleaved caspase-3 in the injured spinal cord.**
(A) After 14 days of continuous treatment with TFHL (5, 10, and 20 mg/kg), the protein expression levels of Bcl-2, Bax, and cleaved caspase-3 were analyzed by western blot assays in each group (n = 6). (B–D) Gray value ratio of protein bands of Bcl-2, cleaved caspase-3, and Bax. After injury, protein expression levels of Bcl-2, Bax, and cleaved caspase-3 increased. After treatment with TFHL, the protein expression level of Bcl-2 increased, while the protein expression levels of Bax and cleaved caspase-3 decreased with increasing drug concentration in the spinal cord injury (SCI) + TFHL groups. *P < 0.05, vs. sham operation group; #P < 0.05, vs. SCI group; †P < 0.05, vs. SCI+5 mg/kg TFHL group. Data are expressed as the mean ± SD (one-way analysis of variance followed by a least-significant difference $post\ hoc$ test). SCI: Spinal cord injury; TFHL: total flavonoids of hawthorn leaves.

Pharmacologically, TFHL has many physiological functions, such as anti-inflammatory, anti-oxidant, and anti-apoptotic properties (Zhang et al., 2017). Furthermore, TFHL has the advantages of being a mild medication and having only minor side effects. Multiple experiments have demonstrated that TFHL is effective in treating cardiovascular disease in animals, but its clinical applications are relatively limited (Wang et al., 2018; Kurkin et al., 2019). In addition, studies of the effects of TFHL on SCI have not been published in or outside China. Therefore, TFHL remains of great research value.

BBB scores can be used to evaluate the neuromotor function of the damaged spinal cord of rats (Chen et al., 2018; Wilkins et al., 2020). In this study, after TFHL treatment, the BBB scores of SCI rats were notably increased at 14 days, indicating that TFHL can alleviate motor function defects after SCI and promote a faster recovery of motor function.

Inflammation is one of the main factors responsible for

damage in secondary SCI (Jian et al., 2020; Wan et al., 2020). After SCI, inflammatory factors can infiltrate and erode tissues to form pathological cavities (O'Connor et al., 2018; Orr et al., 2018). In addition, inflammatory cytokines penetrate nerve cells, destroy cell walls, and cause cytoplasmic rupture, cytoplasmic outflow, and apoptosis (He et al., 2017). The results of hematoxylin-eosin staining and transmission electron microscopy demonstrated that after TFHL treatment, inflammatory cell infiltration was suppressed, the diseased cavities were reduced, and cell morphology was regular, indicating that TFHL can reduce the inflammatory response and restore the spinal cord structure to a certain extent.

After SCI, the functional status of neuronal changes and Nissl bodies are often used as markers of neuronal cell function. Nissl bodies are abundant in neurons with strong metabolic function. When neurons are damaged, Nissl bodies can be reduced, disintegrated, or even disappear. During the recovery, Nissl bodies reappear, increase, and reach normal levels (Hollinshead et al., 2004; Ooigawa et al., 2006). In this study, Nissl staining revealed that Nissl bodies were observably reduced in the SCI group. After treatment with TFHL, the Nissl bodies in the neurons gradually increased in a dose-dependent manner with THFL, indicating that TFHL can restore the functional status of neurons and exert neuroprotective effects.

Apoptosis is an important factor leading to cell death in spinal cord tissue after SCI (Ding et al., 2020; Sun et al., 2020). Apoptosis is regulated by a variety of apoptosis-related genes and proteins, among which Bcl-2 and Bax are common apoptosis-regulating proteins in the Bcl family and have important functions in regulating and inhibiting apoptosis via various pathways (Zhang et al., 2013; Cao et al., 2020). When cells are apoptotic, Bax can adhere to the mitochondrial membrane, promote cytochrome c release, and activate the caspase protein family to induce apoptosis. In contrast to Bax, Bcl-2 inhibits apoptosis by forming a dimer with Bax (Al-Qathama et al., 2017). Bcl-2 protein expression directly determines whether survival or apoptosis occurs (Huang et al., 2017; Or et al., 2020). TUNEL staining and western blot assays showed that after treatment with TFHL, the apoptosis rate in the spinal cord damage area decreased, Bcl-2 protein expression increased, and the expression of Bax and cleaved caspase-3 protein decreased. All of the above changes were dependent on the concentration of TFHL, indicating that TFHL can reduce apoptosis after SCI.

In conclusion, the recovery of motor function was improved after treatment with TFHL in SCI rat models. Exploration of this mechanism indicates that TFHL has a positive effect on the repair of spinal cord tissue, promotes the functional status of neurons, and inhibits apoptosis. Therefore, our preliminary judgment is that TFHL can reduce apoptosis and exert neuroprotective effects to promote the recovery of motor function of rats with SCI. However, there are still many limitations of this study. First, we only conducted in vivo experiments, and the experimental data are relatively limited. Second, although TFHL has a rich variety and high content of flavonoids, only a part of the TFHL content has been isolated, and some elements of TFHL have not been purified and separated. Despite these limitations, this is also the first preliminary exploration of treatment of SCI rats with TFHL, and this study will provide a theoretical basis for investigating the pharmacological value of TFHL and expanding the treatment of SCI.

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