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

Qiong Zhang1, #, Yin Xiong1, Bo Li1, #, Gui-Ying Deng3, Wen-Wen Fu1, Bai-Chuan Cao2, Shao-Hui Zong2, 3, *, Gao-Feng Zeng1, *

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

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
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-inflammation, 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 spinal 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 and SCI operation were as follows: after the T10 segment, the SCI group 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, the T10 segments of the spinal cord tissues of the five groups of rats were separated 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 were analyzed by ImageJ 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/mm² 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 (lysat e+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, Shanghai, China). The protein sample was diluted with a loading buffer containing 20 mg/mL proteinase-k for 15 minutes at 4°C. The protein sample concentration was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). The protein sample was diluted with a loading buffer containing 20 mg/mL proteinase-k for 15 minutes 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-mouse, 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 BBB score of 10, and the least-significant difference test was used to compare differences between two groups. A value of P < 0.05 was considered statistically significant.

TFHL alleviates spinal cord tissue damage in SCI rats
The results of hematoxylin-eosin staining showed that at 40x 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 100x magnification. The SCI group revealed incomplete spinal cord damage and the scores increased in a dose-dependent manner with increasing TFHL concentration. The effect of TFHL was most pronounced on day 14 (P < 0.05; Figure 1).
Though the concentration of TFHL (< 0.05; Figure 2B) significantly decreased in the SCI + 5, 10, and 20 mg/kg TFHL groups (> P < 0.05), and this percentage 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 + TFHL 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 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 SCI group (P < 0.05). Compared with the SCI group, expression levels of Bax and cleaved caspase-3 were significantly decreased, and the Bcl-2 protein expression level increased in the SCI + 5, 10, and 20 mg/kg TFHL 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.
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). 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.

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...
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; Oogawa 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 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.

Acknowledgments: The authors would like to greatly thank the basic laboratory of Guangxi Medical University and the High Level Innovation Team and the Excellent Scholars Program of Guangxi High Education Institutions, China for their support, which was fundamental to the realization of this work.

Author contributions: Study concept and design: GFZ and SHZ; data collection and analysis, drafting and revision of the original manuscript: QZ, YX, BL; study performance and data collection: GYD, WWF, and BCC. All authors approved the final version of the manuscript.

Conflicts of interest: The authors declare that there are no conflicts of interest associated with this manuscript.

Financial support: The study was supported by the National Natural Science Foundation of China, No. 81860231 (to GFZ), the Natural Science Foundation of Guangxi Zhuang Autonomous Region of China, No. 2016GXNSFDA428144 (to SHZ), the Key Research and Development Project of Guangxi Zhuang Autonomous Region of China, No. guikeAB18221021 (to SHZ). The funding sources had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.

Institutional review board statement: All experimental procedures and protocols 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).

Copyright license agreement: The Copyright License Agreement has been signed by all authors before publication.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

Open peer review: Tomoharu Kuboyama, University of Toyama, Japan. Additional file: Open peer review report 1.

References
Abolfazli A, Peyman S, Nima A, Ali S, Serena A, Hamid HM, Mahdii A (2018) Flavonoids profile and antioxidant activity in flowers and leaves of hawthorn species (Crataegus spp.) from different regions of Iran. Int J Food Prop 21:452-470.
Ahuja CS, Wilson JR, Nori S, Kotter MRN, Druschel C, Curt A, Fehlings MG (2017) Traumatic spinal cord injury. Nat Rev Dis Primers 3:17018.
Al-Qathama A, Gibbons S, Prieto JM (2017) Differential modulation of Bax/Bcl-2 ratio and onset of caspase-3/7 activation induced by derivatives of Justicidin B in human melanoma cells A375. Oncotarget 8:95999-96012.
Allen AR (1911) Surgery of experimental lesion of spinal cord equivalent to experience and teamwork on reliability. Multicenter Animal Spinal Cord Injury Study. J Neurotrauma 13:343-359.
Cao F, Lyu X, Dong KF, Fan C, Zhang JJ, Chen K, Zhang Y, Ma BJ, Hou CL, Zhang CH (2020) AMG-102 inhibits proliferation and induces apoptosis of laryngeal squamous cell carcinoma cells by regulating c-Met/PI3K/Akt pathway. Zhonghua Zhong Liu Za Zhi 42:99-104.
Chen KG, Chen LH, Xu RX, Zhang W (2018) Effect evaluation of methylprednisolone plus methylprednisolone division inhibitor-1 on spinal cord injury rats. Childs Nerv Syst 34:1479-1487.
Ding L, Xu J, Yuan C, Teng X, Wu Q M (2020) MiR-7a ameliorates spinal cord injury by inhibiting neuronal apoptosis and oxidative stress. Eur Rev Med Pharmacol Sci 24:11-17.
Dong P, Pan L, Zhang X, Wang X, Jiang M, Chen Y, Duan Y, Wu H, Xu Y, Zhang P, Zhu Y (2017) Hawthorn (Crataegus pinnatifida Bunge) leave flavonoids attenuate atherosclerosis development in apoE knock-out mice. J Ethnopharmacol 198:479-488.
He Z, Zhou Y, Lin L, Wang Q, Khor S, Yao SY, Li J, Zhen Z, Chen J, Gao W, Wu F, Zhang X, Zhang J, Xu HZ, Wang Y, Xiao J (2017) Dl-3-n-butylphthalide attenuates acute inflammatory activation in rats with spinal cord injury by inhibiting microglial TLR4/NF-κB signalling. J Cell Mol Med 21:3010-3022.
Hollinshead WH, Clark SL (2010) The nissl granules of autonomic neurons. J Comp Neurol 62:155-169.

NEURAL REGENERATION RESEARCH | Vol 16 | No. 2 | February 2021 | 355
Research Article

Huang JH, Yin XM, Xu Y, Xu CC, Lin X, Ye FB, Cao Y, Lin FY (2017) Systemic administration of exosomes released from mesenchymal stromal cells attenuates apoptosis, inflammation, and promotes angiogenesis after spinal cord injury in rats. J Neurotrauma 34:3388-3396.

Jalan D, Saini N, Zaidi M, Pallottie A, Elkabes S, Heavy RF (2017) Effects of early surgical decompression on functional and histological outcomes after severe experimental thoracic spinal cord injury. J Neurosurg Spine 26:62-75.

Jian YP, Dong SJ, Xu SS, Fan J, Liu WJ, Shao XW, Li T, Zhao SH, Wang YG (2020) MicroRNA-34a suppresses neuronal apoptosis and alleviates microglia inflammation by negatively targeting the Notch pathway in spinal cord injury. Eur Rev Med Pharmacol Sci 24:1420-1427.

Kurkin VA, Morozova TV, Pravdivtseva DE, Kurkina AV (2019) Quantitative determination of total flavonoids in blood-red Hawthorn leaves. Pharm Chem 15:850-854.

Liu Z, Yang Y, He L, Pang M, Luo C, Liu B, Rong L (2019) High-dose methylprednisolone for acute traumatic spinal cord injury: a meta-analysis. Neurology 93:e841-850.

Ma LY, Liu RH, Xu XD, Yu MQ, Zhang Q, Liu HL (2010) The pharmacokinetics of C-glycosyl flavones of Hawthorn leaf flavonoids in rat after single dose oral administration. Phytomedicine 17:640-645.

Maggio DM, Chatzipanteli K, Masters N, Patel SP, Dietrich WD, Pearse DD (2012) Acute molecular perturbation of inducible nitric oxide synthase with an antisense approach enhances neuronal preservation and functional recovery after contusive spinal cord injury. J Neurotrauma 29:2244-2249.

O’connor G, Jeffrey E, Madorma D, Marcillo A, Abreu MT, Deo SK, Dietrich WD, Maggio DM, Chatzipanteli K, Masters N, Patel SP, Dietrich WD, Pearse DD, Ma LY, Liu RH, Xu XD, Yu MQ, Zhang Q, Liu HL (2010) The pharmacokinetics of C-glycosyl flavones of Hawthorn leaf flavonoids in rat after single dose oral administration. Phytomedicine 17:640-645.

Oliveri RS, Bello S, Biering-Sorensen F (2014) Mesenchymal stem cells improve tissue restoration after traumatic brain injury. J Neurobiol 35:2159-2166.

O’Neill G, Jeffrey E, Madorma D, Marcillo A, Abreu MT, Deo SK, Dietrich WD, Daunert S (2018) Investigation of microbiota alterations and intestinal inflammation post-spinal cord injury in rat model. J Neurotrauma 35:2159-2166.

Ooigawa H, Nawashiro H, Fukui S, Otani N, Osumi A, Toyooka T, Shima K (2006) The fate of Nissl-stained dark neurons following traumatic brain injury in the regulation of apoptosis. Mol Cell Biochem 351:41-58.

P-Reviewer: Kuboyama T; C-Editor: Zhao M; S-Editors: Wang J, Li CH; L-Editors: Kreiner L, Tajima W, Qiu Y, Song LP; T-Editor: Jia Y

P-Reviewer: Kuboyama T; C-Editor: Zhao M; S-Editors: Wang J, Li CH; L-Editors: Kreiner L, Tajima W, Qiu Y, Song LP; T-Editor: Jia Y

Takenaga M, Ohta T, Tokura Y, Hamaguchi A, Nakamura M, Okano H, Igarashi R (2006) Lecithinized superoxide dismutase (PC-SOD) improved spinal cord injury-induced motor dysfunction through suppression of oxidative stress and enhancement of neurotrophic factor production. J Control Release 110:283-289.

Tsai CY, Delgado AO, Weinrauch WJ, Manente N, Levy I, Escalon MX, Bryce TN, Spungen AM (2019) Exoskeleton-assisted walking during acute inpatient rehabilitation leads to motor and functional improvement in persons with spinal cord injury: a pilot study. Arch Phys Med Rehabil doi:10.1016/j.apmr.2019.11.010.

Wang G, An Y, Tao J, Wang Y, Zhou Q, Yang R, Liang Q (2020) MicroRNA-129-5p alleviates spinal cord injury in mice via suppressing the apoptosis and inflammatory response through HMGB1/TLR4/NF-kappaB pathway. Biosci Rep doi:10.1042/BRS20193315.

Wang DJ, Cai YQ, Pan SZ, Zhang LZ, Chen YX, Chen FM, Jin M, Yan MX, Li XD, Chen ZY (2018) Effect of total flavones of Haw leaves on nuclear factor erythroid-2 related factor and other related factors in nonalcoholic steatohepatitis rats. Chin J Integr Med 24:265-271.

Wang J, Li H, Ren Y, Yao Y, Hu J, Zheng M, Ding Y, Chen YY, Shen Y, Wang LL, Zhu Y (2018) Local delivery of beta-elemene improves locomotor functional recovery by alleviating endoplasmic reticulum stress and reducing neuronal apoptosis in rats with spinal cord injury. Cell Physiol Biochem 49:595-609.

Wei Z, Zuo F, Wang W, Wang L, Tong D, Zeng Y, Wang P, Meng X, Zhang Y (2017) Protective effects of total flavones of Elaeagnus rhamnoides (L.) A. nelson against vascular endothelial injury in blood stasis model rats. Evid Based Complement Alternat Therat doi: 10.1155/2017/8142562.

Wen L, Lin Y, Lu R, Yan H, Yu J, Zhao H, Wang X, Wang D (2017) An efficient method for the preparative isolation and purification of flavonoids from leaves of crataegus pinnatifida var. ss. SCCC and -pre-HPLC. Molecules doi:10.3390/molecules22050767.

Wilkins N, Skinner NP, Motovyyakh A, Schmit BD, Kurpad S, Budde MD (2020) Evolution of magnetic resonance imaging as predictors and correlates of functional outcome after spinal cord contusion injury in the rat. J Neurotrauma 37:889-898.

Wu P, Yan X, Z, Zhang Y, Huo DS, Song W, Fang X, Wang H, Yang Y, Jia JX (2018) The protective mechanism underlying total flavones of Dracocephalum (TFD) effects on rat cerebral ischemia-reperfusion injury. J Toxicol Environ Health A 81:1108-1115.

Wu Y, Liu X, Wang W, Zhang HW (2020) Analysis of related factors of deep venous thrombosis after spinal cord injury. Zhongguo Gu Shang 33:140-147.

Yang YG, Yang J, Cheng XH, Wang Z, Zhao BH, Zhao F, Chen ZG, Zhong H (2019) The protection of acute spinal cord injury by subarachnoid space injection of Danshen in animal models. J Spinal Cord Med 42:355-359.

Zhang HX, Zhang K, Wang QG, Shi HK, Wu FZ, Lin BB, Xu XL, Wang XJ, Xu XB, Li ZY, Chen CJ, Li XK, Xiao J (2013) Exogenous basic fibroblast growth factor inhibits ER stress-induced apoptosis and improves recovery from spinal cord injury. CNS Neurosci Ther 19:20-29.

Zhang L, Liu X, Yu H, Zhao R, Liu C, Zhang R, Zhang Q (2019) Comparative pharmacokinetic study on phenolic acids and flavonoids in spinal cord injury rats plasma by UPLC-MS/MS after single and combined oral administration of Danshen and Huangqin extract. J Pharm Biomed Anal 172:103-112.

Zhang P, Holsher C, Ma X (2017) Therapeutic potential of flavonoids in spinal cord injury. Rev Neurosci 28:87-101.

Zhang Q, Liu X, Yan L, Zhao R, An J, Liu C, Yang H (2019) Danshen extract (Salvia miltiorrhiza Bunge) attenuate spinal cord injury in a rat model: a metabolomic approach for the mechanism study. Phytomedicine 62:152966.

Zhang Q, Zhang LX, An J, Yan L, Liu CC, Zhao JY, Huang G (2018) Huangqin flavonoid extraction for spinal cord injury in a rat model. Neural Regen Res 13:2200-2208.

Zhou H, Cheng L, Du X, Hou Y, Liu Y, Cui Z, Nie L (2016) Transplantation of cerebral dopamine neurotranspirig factor transducted bmcs in contusion spinal cord injury of rats: promotion of nerve regeneration by alleviating neuroinflammation. Mol Neurobiol 53:187-199.

Zhou Z, Liu C, Chen S, Zhao H, Zhou K, Wang W, Yuan Y, Li Z, Guo Y, Shen Z, Mei X (2017) Activation of the Nrf2ARE signaling pathway by procubol contributes to inhibiting inflammation and neuronal apoptosis after spinal cord injury. Oncotarget 8:52078-52093.

Zhu Y, Feng B, He S, Su Z, Zheng G (2018) Resveratrol combined with total flavones of Hawthorn alleviate the endothelial cells injury after coronary bypass graft surgery. Phytomedicine 40:20-26.