Erxian decoction inhibits apoptosis by activating Akt1 and repairs spinal cord injury in rats

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ABSTRACT

Objective: Spinal cord injury (SCI) often leads to severe physiological and pathological changes in patients. Erxian Decoction (EXD) is effective in the postoperative treatment of spinal cord injury, but its specific mechanism of action is poorly defined.

Methods: Network pharmacology and molecular docking were used to predict the potential mechanisms of EXD in SCI. In vivo studies were used to validate the above predictions. For in vivo study, the rats were pretreated with or without EXD (5.76 g/kg, by intragastric gavage). Multiple molecular biological test methods to identify molecular mechanisms. One-way analysis of variance (ANOVA) was used with Bonferroni’s post-hoc test to identify the differences between groups.

Results: In vivo studies have shown that EXD improved motor function at 7dpi in SCI rats (P < 0.0001), significantly reduced spinal cord edema (P = 0.0139), upregulated 5-HT, GFAP, and TMEM119 expression. Through network pharmacology analysis, we found that Akt1 in EXD plays a role in treating SCI. The underlying mechanism may be the inhibition of apoptosis after activation of Akt1 phosphorylation. Molecular docking revealed that the key compounds could spontaneously bind to the Akt1 protein. Pharmacological inhibition of Akt1 activation by MK-2206, attenuated the anti-apoptotic effect of EXD on SCI in rats (P < 0.0001).

Conclusions: EXD inhibits apoptosis by activating Akt1, reduce spinal cord edema and restore behavioral function after SCI in rats.

1. Introduction

Spinal cord injury (SCI) refers to structural damage and functional impairment of intraspinal nerves resulting from various factors. Spinal cord function is impaired at and below the level of injury. Traumatic SCI involving an external physical impact that acutely damages the spinal cord [1]. In traumatic SCI, deformation of the spinal cord, inflammation, ischemia, and necrosis of neurons and glial cells, resulting in the formation of cysts and further triggering scar formation, occur at the site of the injury. The lesion area contains several cell types, such as reactive astrocytes, fibroblasts, microglia, and macrophages, which do not undergo spontaneous axonal regeneration [2, 3, 4, 5]. The mechanisms of SCI include a range of biochemical and cellular processes, such as electrolyte abnormalities, free radical formation, vascular ischemia, edema, posttraumatic inflammatory response, apoptosis, and genetically programmed cell death [6]. Apoptosis is a form of programmed cell death, and unlike necrosis, apoptosis is an active process characterized by nuclear condensation, chromatin accumulation, and cell shrinkage [7]. Apoptosis is thought to be a neuronal cell death that can occur during embryonic development [8]. Similarly, apoptosis has been shown to occur in the spinal cord [9], with concomitant activation of caspase-3 apoptotic pathway-related proteins [10]. Therefore, inhibition of

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neuronal cell death may be an effective measure to alleviate spinal cord injury.

Erxian decoction (EXD), which was first introduced by Zhang Bo-Na in the early 1950s [11], is a well-known traditional Chinese medicine (TCM) formulation comprising six herbs: Epimedium brevicornum (Yin Yang Huo), Curculigo orchioides (Xian Mao), Morinda officinalis (Ba Ji Tian), Angelica sinensis (Dang Gui), Anemarrhena asphodeloides (Zhi Mu) and Phellodendron chinense (Huang Bo). Previous studies have shown that EXD can inhibit apoptosis [12], exert neuroprotective effects and ameliorate depression-like behavior in mice [13]. EXD is effective in the postoperative treatment of spinal cord injury, but its specific mechanism of action is poorly defined.

Therefore, this study was designed to explore the role of EXD in SCI as well as the underlying molecular mechanisms.

2. Methods

2.1. Rats, and drugs

Adult female Wistar rats weighing 250 g were purchased from SPF Biotechnology Co., Ltd. (license number: SCXK (Jing) 2019-0010) for the study. Rats were housed together in individually ventilated cages with two or four rats per cage and maintained in an environmentally controlled room (22–24 °C) with a 12-h light/dark cycle and were provided with adequate food and water. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals and complied with ARRIVE guidelines in a licensed laboratory for animal experiments (license number: SYXX (Shaan) 2020-007). The study was reviewed and approved (No. IACUC - 20201003) by the Welfare and Ethics Committee of the Laboratory Animal Center of Air Force Military Medical University, China.

Chinese Herbal Medicine of MED was obtained from China Resources Sanjiu Pharmaceutical Co., Ltd (Table 1). MK-2206, A pharmacological inhibitor of Akt1 activity, was purchased from Medchem Express (Monmouth Junction, USA).

2.2. Surgeries

Long-distance transport of rats may lead to weight loss and mood changes. In order to eliminate the irrelevant factors that affect the experimental results, we routinely feed the rats for 3–5 days, wait for the rats to regain their weight and rule out the anxiety caused by environmental changes, and then start the next experiment.

Random numbers were generated via the standard = RAND () function in Microsoft Excel (version 2019, Microsoft Corp., Redmond, WA, USA). Rats were randomly divided into 4 groups [1]: sham [2]; SCI [3]; SCI + EXD (5.76 g/kg, by intragastric gavage) [4]; SCI + EXD (5.76 g/kg, by intragastric gavage) + MK-2206 (480 mg/kg, once a week). Three independent investigators performed the study. The first investigator grouped the animals according to the random number table. A second investigator, who was uniquely aware of the treatment group assignment, was responsible for the treatment, while a third investigator performed the assessment.

After anesthesia with 25 mg/kg pentobarbital sodium solution, a midline incision was made over the thoracic spine, followed by a T8–T10 laminectomy. According to the results of our previous study, an impactor weighing 10 g was dropped vertically from a height of 50 mm onto the exposed T9 spinal cord surface, causing a contusion injury. Rats were included in the experiment if they presented with a tail swing action, otherwise excluded. Rats in the sham group received only T9 laminectomy without vertical impact. After surgery, the mice were placed on a warming pad until fully awake, and assisted urination (twice a day) was also given until spontaneous urination recovered.

A total of 82 rats were used for the experiment. For each group, six rats were used to test motor function, five rats for immunofluorescence analysis of neural markers, three rats for the determination of spinal cord edema, three rats for the TUNEL staining and three rats for Western blot analysis.

2.3. Network pharmacology

2.3.1. Prediction of active ingredients and targets of EXD

Six herbs, ‘Epimedium brevicornum’, ‘Curculigo orchioides’, ‘Morinda officinalis’, ‘Angelica sinensis’, ‘Anemarrhena asphodeloides’ and ‘Phellodendron chinense’, were used as Herb names to search the active ingredients of EXD in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://lsp.nwu.edu.cn/tcmsp.php). Oral bioavailability (OB) ≥ 30% and druglikeness (DL) ≥ 0.18 were used to screen the active ingredients [14, 15]. The obtained target information was input into Uniprot (https://www.uniprot.org) to obtain the corresponding standard gene name.

2.3.2. SCI target screening

The keywords “SCI”, “Spinal Cord Injury”, and “spinal cord injury” were entered into the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/), DisGeNET (https://www.disgenet.org/), GeneCards (https://www.genecards.org), DrugBank (https://go.drugbank.com/), and PolySearch 2 databases (https://polysearch.cs.ualberta.ca/) to search for the relevant genes. The final SCI target genes were obtained after deduplication of the relevant genes.

2.3.3. Protein-protein interaction network (PPI network)

Intersection target genes were entered into the STRING online database (https://string-db.org/) with the multiple proteins option. The

| Table 1. Pharmaceutical ingredient of Erxian decoction. |
|-----------------------------------------------|
| Herb name (Dosage/300ml) | Herb pinyin name | Properties | Use part | Function | Therapeutic class |
|--------------------------|------------------|-----------|---------|---------|-----------------|
| Curculigo orchioides (10g) | XIAN MAO | Hot; Pungent | Rhizome | To reinforce the kidney yang, to strengthen the tendons and bones, and to dispel cold-damp. | Yang Reinforcing Drugs |
| Epimedium brevicornum (30g) | Yin Yang Huo | Warm; Pungent; Sweet | Epimediumbrevicornum Maxim | Treatment of impotence, seminal emission, weakness of the limbs, rheumatic or rheumatoid arthralgia with numbness and muscle contracture, climacteric hypertension. | For tonifying weakness |
| Phellodendron chinense (12g) | HUANG BO | Cold; Bitter | Bark | 1. To clear heat and dry dampness; 2. To reduce fire and release toxins | Heat-Clearing And Dampness-drying Medicinal |
| Anemarrhena asphodeloides (10g) | ZHI MU | Cold; Sweet; Bitter | Rhizome | To remove heat and quench fire, and to promote the production of body fluid and relieve dryness syndromes. | Fire Purging Drugs |
| Angelica sinensis (10g) | DANG GUI | Warm; Pungent | Root | To enrich blood, activate blood circulation, regulate menstruation, relieve pain, and relax bowels. | Blood-Tonifying Medicinal |
| Morinda officinalis (10g) | BA JI TIAN | N/A | root | N/A | Yang Reinforcing Drugs |
network type was set to full work, and the minimum full score was set to medium confidence (0.400) to obtain PPI network information, which was imported into Cytoscape 3.7.1 to visualize the PPI network.

2.3.4. GO and KEGG pathway enrichment analysis

The intersection target genes were entered into the Metascape online database (http://metascape.org/gp/index), with following setting: Min Overlap: 3, P Value Cutoff: 0.01, Min Enrichment: 1.5. Gene ontology (GO)-related databases and Kyoto encyclopedia of genes and genomes (KEGG) pathway databases were selected, and relevant data were imported into the LC-bio Cloud Platform (https://www.omicstudio.cn). Bar graphs and bubble graphs were plotted.

2.4. Molecular docking validation

The ligand small molecules were found in the PubChem database, and the corresponding 2D structures were drawn using ChemDraw Professional (Version 16.0.1.4), including quercetin (Figure 1a), kaempferol (Figure 1b), and β-sitosterol (Figure 1c). For receptor structure preparation, the 3D structure of the target protein was downloaded from the PDB and subjected to hydrogenation, water removal, and charge addition (Figure 1d). The docking pocket was identified. Compounds with core targets were selected to enter 3D mode to run energy minimization calculations, and then the structures were imported into AutoDock Tools (Version 1.5.6) for molecular docking. Docking patterns of compounds with proteins were analyzed after the end of the run. The docking patterns of compounds with proteins were analyzed and visualized using PyMOL (Version 2.5.2).

2.5. Basso, Beattie & Bresnahan locomotor rating scale

The Basso, Beattie & Bresnahan (BBB) locomotor rating scale was used to assess the locomotor ability of rats at the time of pre-operation and awakening (0 dpi), 1 dpi, 3 dpi, 7 dpi, 14 dpi, and 35 dpi.

Figure 1. Structure of a ligand small molecule and receptor protein. a, 2D structure of quercetin. b, of kaempferol. c, of β-sitosterol. Left 2D, Right 3D. d, 3D structure of the Akt1 (1unq).
BBB scoring system ranges from 0 to 21, with a score of 21 representing normal movement, with lower scores indicating more impaired motor performance [16].

2.6. Detection of spinal cord edema

Rats were anesthetized with 100 mg/kg pentobarbital sodium solution 48 h after SCI and the water contents of the spinal cord were measured as previously reported [17]. Simply, approximately 5 mm (head-end 2.5mm, tail-end 2.5mm) of the spinal cord centered on the injury was removed and weighed on an electronic balance to obtain the initial wet weight (Supplementary Figure S1). After baking at 80 °C for 48 h, the corresponding dry weight was measured as Eq. (1).

\[
\% \text{ Weight} = \frac{A - B}{A}
\]

\%: degree of spinal cord edema; A: wet weight of spinal cord tissue; B: dry weight of spinal cord tissue.

2.7. Perfusion and immunofluorescence

Rats were perfused 7 dpi after SCI. After anesthesia with 100 mg/kg pentobarbital sodium solution, spinal cord tissues were carefully cut, fixed in 4% paraformaldehyde, and then embedded in paraffin blocks for sectioning. Samples were restored by antigen retrieval (sodium citrate antigen retrieval solution, 1:50, Proteintech, USA) after deparaffinization and hydration of the section. Then, the samples were blocked with ready-to-use normal sheep serum (Boster Biological, China) containing 5% sheep serum albumin in phosphate-buffered saline (PBS) for 1 h at room temperature and then immunostained with primary antibody overnight at 4 °C. The primary antibodies used were: Rabbit anti-5-HT (Immunostar (20080/1907001), 1:1000), Mouse anti-GFAP (Abcam (ab68428), 1:200), and Mouse anti-TMEM119 (Proteintech (66948-1-lg), 1:200). On the following day, the sections were reincubated with secondary antibodies, the secondary antibodies included: Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (H + L) (SA00009-1, Proteintech, 1:200) and CoraLite488-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (SA00013-2, Proteintech, 1:200). The samples were mounted (DAPI Fluoromount-G, Southern Biotech, USA) and imaged with a confocal laser scanning microscope (Olympus BX51) and DP Controller software (Olympus, Japan).

2.8. TUNEL staining

Spinal cord apoptosis was examined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the TUNEL Apoptosis Detection Kit (Roche 11684795910 at 7 dpi after SCI, Roche Diagnostics GmbH Roche Applied Science Mannheim, Germany) according to the manufacturer’s instructions.
| EXD Composition | MOL ID | Group | Molecule name | OB (%) | DL (%) |
|-----------------|--------|-------|---------------|--------|--------|
| Curculigo orchioides | MOL000358 | A1 | Beta-sitosterol | 36.91 | 0.75 |
| Angelica sinensis | MOL000358 | A2 | Beta-sitosterol | 36.91 | 0.75 |
| Morinda officinalis | MOL000358 | A3 | Beta-sitosterol | 36.91 | 0.75 |
| Anemarrhena asphodeloides | MOL001677 | ZM1 | Asperglaucide | 58.02 | 0.52 |
| Anemarrhena asphodeloides | MOL003773 | ZM2 | Mangiferolic acid | 36.16 | 0.84 |
| Anemarrhena asphodeloides | MOL004489 | ZM3 | Anemarsaponin F | 60.06 | 0.79 |
| Anemarrhena asphodeloides | MOL004492 | ZM4 | Chrysanthemeaxanthin | 38.72 | 0.58 |
| Anemarrhena asphodeloides | MOL004497 | ZM5 | Hippeastrine | 51.65 | 0.62 |
| Anemarrhena asphodeloides | MOL004514 | ZM6 | Timosaponin B | 35.26 | 0.87 |
| Anemarrhena asphodeloides | MOL004528 | ZM7 | Icarin I | 41.58 | 0.61 |
| Anemarrhena asphodeloides | MOL004540 | ZM8 | Anemarsaponin C | 35.5 | 0.87 |
| Anemarrhena asphodeloides | MOL004542 | ZM9 | Anemarsaponin E | 30.67 | 0.86 |
| Anemarrhena asphodeloides | MOL000483 | ZM10 | (2)-3-(4-hydroxy-3-methoxy-phenyl)-N-[2-(4-hydroxyphenyl)ethyl]acrylamide | 118.35 | 0.26 |
| Anemarrhena asphodeloides | MOL000546 | ZM11 | Diosgenin | 80.88 | 0.81 |
| Anemarrhena asphodeloides | MOL000631 | ZM12 | Coumaroyltyramine | 112.9 | 0.2 |
| Phellodendron chinense | MOL000358 | B4 | Beta-sitosterol | 36.91 | 0.75 |
| Curculigo orchioides | MOL000449 | B1 | Stigmasterol | 43.83 | 0.76 |
| Curculigo orchioides | MOL001607 | XM1 | ZINC03982454 | 36.91 | 0.76 |
| Curculigo orchioides | MOL003578 | XM2 | Cycloartenol | 38.69 | 0.78 |
| Angelica sinensis | MOL000449 | B2 | Stigmasterol | 43.83 | 0.76 |
| Curculigo orchioides | MOL004114 | XM3 | 3,2',4',6'-Tetrahydroxy-4,3'-dimethoxy chalcone | 52.69 | 0.28 |
| Curculigo orchioides | MOL004125 | XM4 | Curculigoside B | 83.36 | 0.19 |
| Phellodendron chinense | MOL000449 | B3 | Stigmasterol | 43.83 | 0.76 |
| Epimedium brevicornum | MOL001510 | XLP1 | 24-epicampsterol | 37.58 | 0.71 |
| Epimedium brevicornum | MOL001645 | XLP2 | Linoleyl acetate | 42.1 | 0.2 |
| Epimedium brevicornum | MOL001792 | XLP3 | DFV | 32.76 | 0.18 |
| Epimedium brevicornum | MOL003044 | XLP4 | Chryseriol | 35.85 | 0.27 |
| Epimedium brevicornum | MOL003542 | XLP5 | 8-Isopentenyl-kaempferol | 38.04 | 0.39 |
| Epimedium brevicornum | MOL004367 | XLP6 | olivil | 62.23 | 0.41 |
| Epimedium brevicornum | MOL004380 | XLP7 | C-Homoerythrinan, 1,6-didehydro-3,15,16-trimethoxy-, (3.beta.) | 39.14 | 0.49 |
| Epimedium brevicornum | MOL004382 | XLP8 | Yinyanghuo A | 56.96 | 0.77 |
| Epimedium brevicornum | MOL004384 | XLP9 | Yinyanghuo C | 45.67 | 0.5 |
| Epimedium brevicornum | MOL004386 | XLP10 | Yinyanghuo E | 51.63 | 0.55 |
| Epimedium brevicornum | MOL004388 | XLP11 | 6-hydroxy-11,12-dimethoxy-2,2-dimethyl-1,8-dioxo-2,3,4,8-tetrahydro-1H-isochromeno[3,4-h]isoquinolin-2-isam | 50.64 | 0.17 |
| Epimedium brevicornum | MOL004391 | XLP12 | 8-(3-methylbut-2-enyl)-2-phenyl-chromone | 48.54 | 0.25 |
| Epimedium brevicornum | MOL004394 | XLP13 | Anhydroicaritin-3-O-alpha-L-rhamnoside | 41.58 | 0.61 |
| Epimedium brevicornum | MOL004396 | XLP14 | 1,2-bis(4-hydroxy-3-methoxyphenyl)propan-1,3-diol | 52.31 | 0.22 |
| Epimedium brevicornum | MOL004425 | XLP15 | Icarin | 41.58 | 0.61 |
| Epimedium brevicornum | MOL004427 | XLP16 | Icariside A' | 31.91 | 0.86 |
| Epimedium brevicornum | MOL000006 | XLP17 | Luteolin | 36.16 | 0.25 |
| Anemarrhena asphodeloides | MOL000449 | B4 | Stigmasterol | 43.83 | 0.76 |
| Epimedium brevicornum | MOL001771 | C1 | Poriferast-5-en-3beta-ol | 36.91 | 0.75 |
| Epimedium brevicornum | MOL00359 | D1 | Sitosterol | 36.91 | 0.75 |
| Morinda officinalis | MOL00359 | D2 | Sitosterol | 36.91 | 0.75 |
| Epimedium brevicornum | MOL000422 | E1 | Kaempferol | 41.88 | 0.24 |
| Anemarrhena asphodeloides | MOL000422 | E2 | Kaempferol | 41.88 | 0.24 |

(continued on next page)
| EXD Composition | MOL ID | Group | Molecule name | OB (%) | DL (%) |
|-----------------|--------|-------|---------------|--------|--------|
| Morinda officinalis | MOL001506 | BJT1 | Suraene | 33.55 | 0.42 |
| Morinda officinalis | MOL002879 | BJT2 | Diop | 43.59 | 0.39 |
| Morinda officinalis | MOL002883 | BJT3 | Ethyl oleate (NF) | 32.4 | 0.19 |
| Epimedium brevicornum | MOL004373 | F1 | Anhydroicaritin | 45.41 | 0.44 |
| Morinda officinalis | MOL006147 | BJT4 | Alizarin-2-methylether | 32.81 | 0.21 |
| Morinda officinalis | MOL009495 | BJT5 | 2-Hydroxy-1,5-dimethoxy-6-(methoxymethyl)-9,10-anthraquinone | 95.85 | 0.37 |
| Morinda officinalis | MOL009496 | BJT6 | 1,5,7-Trihydroxy-6-methoxy-2-methoxymethylanthracenequinone | 80.42 | 0.38 |
| Morinda officinalis | MOL009500 | BJT7 | 1,6-Dihydroxy-5-methoxy-2-(methoxymethyl)-9,10-anthraquinone | 104.54 | 0.34 |
| Morinda officinalis | MOL009503 | BJT8 | 1-Hydroxy-3-methoxy-9,10-anthraquinone | 104.33 | 0.21 |
| Morinda officinalis | MOL009504 | BJT9 | 1-hydroxy-6-hydroxymethylanthracenequinone | 81.77 | 0.21 |
| Morinda officinalis | MOL009513 | BJT10 | 2-hydroxy-1,8-dimethoxy-7-methoxymethylanthracenequinone | 112.3 | 0.37 |
| Morinda officinalis | MOL009524 | BJT12 | 2beta,20(R),5-alkenyl-stigmastol | 36.91 | 0.75 |
| Morinda officinalis | MOL009525 | BJT13 | 3beta-24S(R)-butyl-5-alkenyl-cholestol | 26.35 | 0.82 |
| Morinda officinalis | MOL009537 | BJT14 | americanin A | 46.71 | 0.35 |
| Morinda officinalis | MOL009541 | BJT15 | Asperuloside tetraacetate | 45.47 | 0.82 |
| Morinda officinalis | MOL009551 | BJT16 | Isoprinocarpine | 49.12 | 0.77 |
| Morinda officinalis | MOL009558 | BJT17 | 2-hydroxyethyl 5-hydroxy-2-(2-hydroxybenzoyl)-4-(hydroxymethyl)benzofuran | 62.32 | 0.26 |
| Morinda officinalis | MOL009562 | BJT18 | Chelerythrine | 34.18 | 0.78 |
| Anemarrhena asphodeloides | MOL004373 | F2 | Anhydroicaritin | 45.41 | 0.44 |
| Epimedium brevicornum | MOL000622 | G1 | Magnograndiolide | 63.71 | 0.19 |
| Phellodendron chinense | MOL000622 | G2 | Magnograndiolide | 63.71 | 0.19 |
| Phellodendron chinense | MOL001454 | HB1 | Berberine | 36.86 | 0.78 |
| Phellodendron chinense | MOL001458 | HB2 | Coptisine | 30.67 | 0.86 |
| Phellodendron chinense | MOL002636 | HB3 | Khadalactone A | 34.21 | 0.82 |
| Phellodendron chinense | MOL001352 | HB4 | Obtusunone | 43.29 | 0.77 |
| Phellodendron chinense | MOL002641 | HB5 | Phellavin,qt | 35.86 | 0.44 |
| Phellodendron chinense | MOL002643 | HB6 | delta 7-stigmasterol | 37.42 | 0.75 |
| Phellodendron chinense | MOL002644 | HB7 | Phellopterin | 40.19 | 0.28 |
| Phellodendron chinense | MOL002651 | HB8 | Dehydrotaxanthinol A | 43.76 | 0.4 |
| Phellodendron chinense | MOL002652 | HB9 | delta7-Dehydrotaxanthinol | 54.45 | 0.25 |
| Phellodendron chinense | MOL002656 | HB10 | Dihydroniloticin | 36.43 | 0.81 |
| Phellodendron chinense | MOL002659 | HB11 | khadalin A | 31.6 | 0.7 |
| Phellodendron chinense | MOL002660 | HB12 | Nilotin | 41.41 | 0.82 |
| Phellodendron chinense | MOL002662 | HB13 | Rutacarpine | 40.3 | 0.6 |
| Phellodendron chinense | MOL002663 | HB14 | Skimmianin | 40.14 | 0.2 |
| Phellodendron chinense | MOL002666 | HB15 | Chelerythrine | 34.18 | 0.78 |
| Phellodendron chinense | MOL002668 | HB16 | Worenine | 45.83 | 0.87 |
| Phellodendron chinense | MOL002670 | HB17 | Cavidine | 35.64 | 0.81 |
| Phellodendron chinense | MOL002671 | HB18 | Candeltoxin A | 31.81 | 0.69 |
| Phellodendron chinense | MOL002672 | HB19 | Hericenone H | 39 | 0.63 |
| Phellodendron chinense | MOL002673 | HB20 | Hispidone | 36.18 | 0.83 |
| Phellodendron chinense | MOL000762 | HB21 | Palmidin A | 35.36 | 0.65 |
| Phellodendron chinense | MOL000785 | HB22 | Palmitine | 64.6 | 0.65 |
| Phellodendron chinense | MOL000787 | HB23 | Fumarine | 59.26 | 0.83 |
| Phellodendron chinense | MOL000790 | HB24 | Iosoropylmine | 35.77 | 0.59 |
| Phellodendron chinense | MOL001131 | HB25 | phellamurin,qt | 56.6 | 0.39 |
| Phellodendron chinense | MOL001455 | HB26 | (S)-Canadine | 53.83 | 0.77 |
| Phellodendron chinense | MOL002894 | HB27 | Berberrubine | 35.74 | 0.73 |
| Phellodendron chinense | MOL005438 | HB28 | Campesterol | 37.58 | 0.71 |
| Phellodendron chinense | MOL006392 | HB29 | dihydroniloticin | 26.43 | 0.82 |
| Phellodendron chinense | MOL006401 | HB30 | Melianone | 40.53 | 0.78 |
| Phellodendron chinense | MOL006413 | HB31 | Phellochin | 35.41 | 0.82 |
| Phellodendron chinense | MOL006422 | HB32 | Thalidomide | 44.41 | 0.73 |
| Epimedium brevicornum | MOL000998 | H1 | Quercetin | 46.43 | 0.28 |
| Phellodendron chinense | MOL000998 | H2 | Quercetin | 46.43 | 0.28 |
2.9. Western blot analysis

Rat spinal cord tissue (dissociated at 7dpi post-SCI) was lysed in the denaturing buffer of the Minute™ Total Protein Extraction Kit (Cat# SD-001, Invent Biotechnologies, Beijing, China) to obtain protein extracts. The protein concentration of the supernatant was detected using a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) with a full-wavelength functional microplate reader (Infinite M200Pro, Tecan, Switzerland). Proteins (30 μg/sample) were separated using 12.5% SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 10% nonfat dry milk for 1 h, phosphorylated proteins were blocked using bovine serum albumin (5% BSA, room temperature) for 2 h. The membranes were incubated overnight at 4 °C with the following primary antibodies: Bel-2 (1:500, Affinity Biosciences); cleaved caspase-3 (Asp175) (1:1000, Cell Signaling Technology); BAX (1:1000, Proteintech); Akt1 (1:1000; Abcam ab233755); Anti-Akt1 (phosphor S473, 1:4000, Abcam, ab81283); β-Actin (1:6000; Signalway Antibody). Appropriate HRP-conjugated secondary antibodies (1:80,000, Abmart, Shanghai, China) were applied for 1 h at room temperature. After 3 PBS-T washes as

Figure 3. Network pharmacology analysis. a, Gene network diagram of 6 herbs screened from the TCMSP database. b, Wayne diagram showing that a total of 1903 genes were associated with SCI and 250 genes were associated with the drugs, with 151 key genes.
described above three times, the membranes were visualized using the ECL Western Blot Detection Kit (Merck, USA). Analysis was performed using the Bole CHEMIDOCXRS chemiluminescence imaging system and QuantityOne software.

2.10. Statistical analysis

Normality of distribution and equality of variances were tested by SPSS (Version 25.0). One-way analysis of variance (ANOVA) was used to
process more than two groups of data. Multiple comparisons were carried out to identify the differences between groups using Bonferroni’s post-hoc test. The error bars in all figures represent the mean ± standard error of the mean (SEM). \( P < 0.05 \) was regarded as statistically significant. All data were analyzed using SPSS and GraphPad Prism (version 6.0c).
therefore the most stable (Figure 5a, b, c) (Table 3). EXD treatment group compared with the SCI group (Figure 6a). MK-2206 performed, and the results showed effectively inhibited apoptosis in the regulating apoptosis. To test the above hypothesis, TUNEL staining was above, it is hypothesized that EXD mediates SCI repair by negatively 3.3. EXD inhibiting apoptosis of SCI by activating Akt1 molecules in rats docks freely with Akt1, and the key amino acids around it mainly func-

| molecular docking  | Putative target | Run | Binding energy (kcal/mol) |
|---------------------|-----------------|-----|--------------------------|
| kaempferol          | Akt1            | 3/50| −7.27                    |
| beta-sitosterol     | Akt1            | 7/50| −7.22                    |
| quercetin           | Akt1            | 8/50| −4.65                    |

3. Results

3.1. EXD can effectively repair function after SCI in rats

To investigate the effectiveness of EXD in the treatment of SCI in rats, the behavioral performance of BBB score grading at 0, 1, 3, 7, 14 and 35 dpi after SCI was observed. The sham group did not suffer from motor impairment, whereas the remaining two groups showed severe hindlimb dyskinesia. There were not significantly different at 3 dpi (P = 0.0139), but significant statistical differences appeared at 7 dpi, 14 dpi, and 35 dpi (P < 0.0001) (Figure 2a).

Subsequent immunoﬂuorescence showed that EXD treatment signiﬁcantly increased the expression of 5-HT (P < 0.0001), GFAP (P < 0.0001), and TMEM119 (P = 0.0041) around the injury site. Studies have shown that EXD treatment increased the expression of spinal cord-related cell marker genes, decreased the degree of spinal cord edema and improved mobility in SCI rats (Figure 2d, e, f, g).

3.2. Network pharmacology screens potential molecular targets of EXD in SCI

To explore the possible molecular mechanism of EXD in SCI, a total of 103 active components of EXD were screened, among which quercetin, β-sitosterol and kaempferol had a significant effect (Table 2). A total of 250 target genes of EXD (some active ingredients had no target) were predicted by the TCMSP database and UniProt (Figure 3a). There were 1903 SCI target genes, which were mapped with the intersection of EXD target genes, resulting in a total of 151 target genes for the intersection of EXD treatment with SCI (Figure 3b). We construct the PPI network diagram of target genes of EXD-SCI. A total of 17 key genes, such as Akt1, were selected by selecting DC > 34 through the CytoNCA plugin to select the secondary network and further through DC > 62, BC > 36, and CC > 0.85 (Figure 4a). The biological processes involved in EXD-SCI mainly included negative regulation of the extrinsic apoptotic signaling pathway and mainly included the PI3K-Akt signaling pathway and the TNF signaling pathway and so on (Figure 4b, c).

Based on the above screening results, the 3D structure of Akt1 was imported into AutoDock Tools and docked with quercetin, β-sitosterol and kaempferol. Molecular docking results showed that kaempferol docks freely with Akt1, and the key amino acids around it mainly function in the form of hydrogen bonds. The formation of hydrogen bonds minimizes the energy of the small molecule–receptor complex and is therefore the most stable (Figure 5a, b, c) (Table 3).

3.3. EXD inhibiting apoptosis of SCI by activating Akt1 molecules in rats

According to the results of the biological process analysis described above, it is hypothesized that EXD mediates SCI repair by negatively regulating apoptosis. To test the above hypothesis, TUNEL staining was performed, and the results showed effectively inhibited apoptosis in the EXD treatment group compared with the SCI group (Figure 5a). MK-2206 [18,19] is a potent, selective, allosteric Akt inhibitor that promotes apoptosis and decreases Akt phosphorylation. We found that the anti-apoptotic effect of EXD may lie in the induction of Akt1 phosphorylation at position Ser473. The results showed that EXD treatment significantly inhibited apoptosis after SCI in rats. However, pharmacological inhibition of Akt1 activation by MK-2206, attenuated the antiapoptotic effect of EXD on SCI in rats (P < 0.0001). Phosphorylation of Akt1 can be activated by EXD (Figure 6b, c) (P < 0.0001). Decreased cleaved caspase-3 and Bax levels, as well as increased Bcl-2 levels, were detected in the EXD group compared to the SCI group; however, when phosphorylated Akt1 was inhibited, the antiapoptotic effect of EXD was significantly alleviated (Bax to Bcl2, P = 0.006; cleaved caspase 3 to β-actin, P < 0.0001) (Figure 6b, d, e).

4. Discussion

The main objective of this study was to investigate the effect of EXD on functional repair after SCI as well as the underlying molecular mechanisms. This study found that EXD significantly increased the behavioral scores of the rats, reduced spinal cord edema, inhibited apoptosis by activating Akt1 phosphorylation, and regulated the expression of the apoptosis-related proteins BAX, BCL-2, and cleaved caspase 3.

Multiple compounds were clearly detected in rat brain at 4 h after oral EXD administration, suggesting that some of the original compounds contained in EXD can pass through the blood-brain barrier and act directly on the central nervous system [20]. EXD significantly increases cell viability, exerts an antidepressant-like effect on behavior, and has neuroprotective potential [13]. Among the six herbs comprising EXD, *Epimedium brevicornum* is neuroprotective and can reduce neuro-inflammation in rats [21]. The neuroprotective effect of curcugioside, a major component of *C. officinalis*, is associated with downregulation of the expression of apoptosis-related proteins and a reduction in intracellular reactive oxygen species production [22]. *M. officinalis* has antioxidant, immunomodulatory, and estrogenic activities [23]. *A. sinensis* ameliorates epididymal peroxidation and oxidative stress and inhibits apoptosis [24]. AAP70-1, a novel polysaccharide isolated from *A. asphodeloides*, has potential as a therapeutic agent for central nervous system diseases or as an immunomodulator [25]. *P. chinense* has an excellent inhibitory effect on the overproduction of proinflammatory mediators in vitro [26]. Our data showed that EXD significantly improved the locomotor function of SCI rats, reduced spinal cord edema, and increased the expression of neural cell markers.

In order to determine whether EXD has anti-inflammatory effects, we tried to use RNA-seq technology to explain the expression of inflammatory factors between different groups (GSE174549). The results showed that SCI signiﬁcantly activated a variety of inflammatory factors, and EXD could signiﬁcantly reduce the inflammatory factors activated by SCI, such as Pgf, Ilr2 and TGF-β (Supplementary Figure S2). Ilr2 expression of neutralis mediates the pro-inflammatory effect of Il-1 [27]. TGF-β 1 not only has pro-inflammatory effect [28], but also induces ﬁbroblast activation and promotes cell ﬁbrosis [29]. Pgf is associated with neuroinflammatory plaques [30] and further promotes apoptosis by inducing autophagy [31].

Network pharmacology and molecular docking revealed that β-sitosterol (*C. officinalis, A. sinensis, M. officinalis* and *P. chinense*), kaempferol (*E. brevicornum* and *A. asphodeloides*) and quercetin (*E. brevicornum* and *P. chinense*) are the main active components of EXD (Figure 3a). Molecular docking suggested that Akt1 may be a potential target of β-sitosterol, kaempferol and quercetin (Figure 5a, b, c). Our results indicated that Akt1 may promote spinal cord repair by inhibiting apoptosis (Figure 4b), and the PI3K-Akt signaling pathway may be the pathway underlying these effects (Figure 4c).

In the apoptosis pathway, Bcl-2 family proteins are released to tightly regulate the activation of asparaginase (the main executor of apoptosis), which acts by inducing the release of caspase activators from mitochondria [32]. Bax is involved in the formation of mitochondrial outer membrane pores, leading to the release of the contents of the mitochondrial intermembrane space into the cytoplasm, while high expression of Bcl-2 blocks cell death by preventing Bax activation and
homo-oligomerization [33]. Herein, we used Western blot analysis to assess apoptosis. Our results indicated that EXD increased the prosurvival Bcl-2/Bax ratio and suppressed cleaved caspase-3 expression in spinal cord cells after SCI (Figure 6b, d, e).

We explored the mechanism by which EXD alleviates SCI and found that Akt1 phosphorylation is a key mechanism in EXD-mediated SCI repair. EXD induced an increase in Akt1 phosphorylation. MK-2206, A pharmacological inhibitor of Akt1 activity, inhibits the allosteric activation of AKT [34] and blocks Akt1 phosphorylation. These results suggest that Akt1 is necessary for the EXD-mediated inhibition of apoptosis in SCI cells. Our data strongly suggested that EXD plays a critical role in the inhibition of apoptosis induces by SCI by activating Akt1 phosphorylation. There is a limitation in that we were unable to determine whether other members of the Akt family are involved in the effects of EXD on SCI-induced apoptosis. Further investigation is required to determine the mechanism.

5. Conclusions

In summary, our study demonstrates that EXD plays a role in the inhibition of apoptosis induced by SCI. We identified a novel mechanism that EXD attenuated apoptosis through activating Akt1 phosphorylation, which suggested that EXD might be a promising therapeutic agent for SCI.

Declarations

Author contribution statement

Erliang Li: Performed the experiments; Wrote the paper.
Rongbao Yan: Analyzed and interpreted the data; Wrote the paper.
Kang Yan; Ruqin Huang: Performed the experiments.
Rui Zhang: Analyzed and interpreted the data.
Yanhua Wen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Shuang Li: Contributed reagents, materials, analysis tools or data.
Peng Li; Qiong Ma; Bo Liao: Conceived and designed the experiments.

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Data availability statement

Data associated with this study has been deposited at Gene Expression Omnibus (GEO) database under the accession number GSE174549.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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