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Original Article

Mechanism of Huoxue Tongluo Decoction in treatment of erectile dysfunction caused by ischemic stroke based on network pharmacology

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ABSTRACT

Objective: To study the therapeutic effect of Huoxue Tongluo Decoction (HXTLD) on erectile dysfunction caused by ischemic stroke and identify the mechanisms involved.
Methods: Network pharmacology was used to predict the key active ingredients and targets of HXTLD. Surgical methods were used to create a rat model of ischemic stroke. The rats were then given a suspension of HXTLD by ig administration. Erectile function was evaluated by Apomorphine (APO) induction. Real-time fluorescence quantitative reverse transcription-polymerase chain reaction (Real-time PCR) and Western blotting were used to detect the expression of related mRNAs and proteins in rat penile corpus cavernous tissue and brain tissue. Hematoxylin & Eosin (HE) staining was used to investigate structural changes in the penile cavernous tissue.
Results: Network pharmacology showed that tumor necrosis factor (TNF), nitric oxide synthase 3 (eNOS), and vascular endothelial growth factor (VEGF) were the key targets of HXTLD in the treatment of erectile dysfunction caused by ischemic stroke. Experimental studies showed that HXTLD improved erectile dysfunction caused by ischemic stroke. HE results showed that HXTLD improved the structure of the corpus cavernosa. HXTLD also inhibited the expression of TNF and VEGF proteins in penile tissue ($P < 0.05$) and enhanced the expression of eNOS protein in penile tissue ($P < 0.05$).
Conclusion: HXTLD improved the erectile function of rats with erectile dysfunction caused by ischemic stroke by regulating the mRNA and protein levels of TNF, eNOS and VEGF.

1. Introduction

Ischemic stroke (IS) is a general term used to describe brain tissue necrosis caused by stenosis or occlusion of the arteries supplying the brain (carotid and vertebral arteries) thus causing an insufficient blood supply to the brain. Cerebral ischemia can be divided into localized cerebral ischemia and diffuse cerebral ischemia (Liu & Dai, 2017). Erectile dysfunction (ED) refers to the inability of the penis to maintain a sufficient erection to complete satisfactory sexual intercourse for over a three-month period (Xie, 2016). Epidemiological investigations have found that 48%–75% of male IS patients report ED within 3–24 months after the onset of IS. Furthermore, 17%–42% of IS patients report a loss of libido (Tsertsvadze, Fink & Yazdi, 2009). Following IS, disabled patients also report a significant reduction in sexual desire, sexual satisfaction, the frequency of sexual intercourse, and orgasm (Tsertsvadze, Fink & Yazdi, 2009). In a previous epidemiological study, we investigated the erectile function and sexual activity of 248 male patients with IS and found that 45.56% of these patients had ED within the 6 months prior to the onset of IS and that 77.73% of patients were diagnosed with ED. A significant number of patients ($P < 0.01$) developed ED within 6 months of IS (Ma, 2017). Of the 98 patients with ED prior to the onset of IS (excluding the sexual life after IS), the international index of erectile function (IIEF-5) score dropped from 16.24 points (IQR: 14.75–18) to 9.5 points (IQR: 6.75–12) after IS. We also found that low libido was common after IS and that the frequency of sexual intercourse decreased significantly ($P < 0.01$) from 3 times prior to onset (median, IQR: 2–4) to 1.25 times after onset (median, IQR: 0.5–2) (Ma, 2017). However, the specific mechanisms responsible for the relationship between IS and ED remains unclear. Upon searching the literature, we found that although there are related reports on

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the relationship between IS and ED, most of these existing reports are based on population studies, epidemiological analyses, and clinical research investigations. Therefore, there is an urgent need to identify the mechanistic association between IS and ED by adopting an experimental approach.

Phosphodiesterase type 5 inhibitors (PDE5Is) are considered to be the first-line treatment for ED. These can prevent the degradation of cyclic guanosine monophosphate and can improve erectile function in patients as an alternative treatment option (Yuan, Zhang & Yang, 2013). However, compared with patients with ED without IS, patients with IS-induced ED (ISED) do not respond well to PDE5 inhibitors (Shindel, 2012). Traditional Chinese medicine (TCM) is an important aspect of China's excellent traditional culture. TCM can be used in conjunction with drugs to activate the blood, dredge collaterals, nourish the kidney, and sooth the liver, and can significantly reduce adverse reactions while enhancing the therapeutic effect of ED (Lee, Chen & Shih, 2014; Lin, et al., 2018). Huoxue Tongluo Decoction (HXTLD) is a andrology prescription provided by the Dongzhimen Hospital of Beijing University of Chinese Medicine, has been used widely in clinics for many years, and has a good clinical effect on the treatment of complex ED. This decoction activates the blood to alleviate blood stasis, dredges collaterals, invigorates the kidneys, and provides warming yang (Li, 2019).

Network pharmacology is based on the "disease-gene-target-drug" interaction network and uses network analysis methods to investigate the intervention and impact of drugs on a disease network. This method can also be used to determine the synergy between multiple drugs. It can also combine various disease target databases, verify the molecules associated with a particular disease, and provide evidence for molecular targets and their mechanism of action (Zhang, et al., 2020).

In this study, we combined network pharmacology with experiments in an animal model. First, we used network pharmacology to predict key active ingredients and targets. Second, we investigated the effects of HXTLD in a rat model of ED caused by IS (ISED) and attempted to identify the mechanisms involved.

2. Materials and methods

2.1. Network pharmacology

2.1.1. Extraction of chemical ingredients and the screening of active ingredients

HXTLD contains 12 types of traditional Chinese medicines: leech, centipede, *Paeoniae Radix Rubra*, *Achyranthis Bidentatae Radix*, *Angelicae Sinensis Radix*, *Vaccariae Semen*, *Curcumae Radix*, *Citri Reticulatae Pericarpium Viride*, *Bupleuri Radix*, *Tribuli Fructus*, *Epimrdii Herba*, and *Morindae Officinalis Radix*. Next, we acquired the chemical components of these 12 traditional Chinese medicines from the TCM System Pharmacology Database (TCMSP, https://tcmspw.com/tcmsp.php). We then used drug combination-application similarity (DL) (DL $\geq$ 0.18) and oral bioavailability (OB) (OB $\geq$ 30%) to screen and identify the active ingredients of HXTLD (Liu, et al., 2019).

2.1.2. Target acquisition

Targets for IS and ED were acquired using the Human Gene Database (GeneCards, https://www.genecards.org/). Relevant targets for the active ingredients of HXTLD were acquired from TCMSP, PubChem, and Swiss Target Prediction. We then identified the targets that were common to IS, ED, and HXTLD; These were considered as the potential targets of HXTLD for the treatment of ISED and were used in subsequent analysis to construct a target network (Lisa, Welzel & Friederike, 2019).

2.1.3. Protein-protein interaction (PPI)

Next, we used the STRING database (https://string-db.org/) to identify potential protein–protein interactions (PPI) (Wang, et al., 2019). In order to improve the reliability of the data obtained, PPIs were filtered; The minimum interaction score was set to 0.40. Filtered PPIs were then used for network construction and analysis.

2.1.4. Network construction and analysis

Cytoscape software (version 3.7.1) was used to construct herbal-active ingredient-target-disease network and PPI network. After the network was constructed, we used a network analyzer to calculate the "degree" and other parameters. In addition, the Cytoscape plug-in cytohubba was used to further analyze the PPI network to identify key targets (Hua, Yong, Ma, 2019).

2.1.5. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis

Next, we imported the relevant targets of HXTLD for the treatment of ISED into the DAVID bioinformatics annotation database (https://david.ncifcrf.gov/, version 6.8); We set 'Select Identifier' to OFFICIAL GENE SYMBOL, set the 'List Type Set' to Gene List and set 'species' set to human. The outcomes of GO analysis and KEGG pathway analysis for the use of HXTLD to treat ISED were then saved (Wang, et al., 2021).

2.2. In vivo experimental research

2.2.1. Drugs and reagents

HXTLD were purchased from Kangrentang (Beijing, China). The specific reagents were as follows: leech (6 g), centipede (3 g), *Paeoniae Radix Rubra* (20 g), *Achyranthis Bidentatae Radix* (15 g), *Angelicae Sinensis Radix* (15 g), *Vaccariae Semen* (10 g), *Curcumae Radix* (10 g), *Citri Reticulatae Pericarpium Viride* (10 g), *Bupleuri Radix* (15 g), *Tribuli Fructus* (20 g), *Epimrdii Herba* (10 g), and *Morindae Officinalis Radix* (15 g). These reagents were ground into fine powder and dissolved in deionized water. We also acquired a range of antibodies: tumor necrosis factor (TNF) antibody (Bioss, lot number: Bs-10802R), nitric oxide synthase 3 (eNOS) antibody (Abcam, lot number: ab76198), Vascular Endothelial Growth Factor (VEGF) antibody (Abcam, lot number: ab1316), and β-actin antibody (Abcam, lot number: ab6276).

2.2.2. Preparation of experimental animals

Thirty SPF-grade adult male SD rats (7 weeks old, mean weight: 200 ± 20 g) were purchased from Beijing Weitonglihuaxian Animal Technology Co., Ltd. (Certificate number: SYXK (Beijing) 2016–0006, Beijing, China). The rats were reared in a clean environment at a constant temperature of 22–24 °C. All rats were adaptively fed for 1 week prior to experiments. The experimental protocol was approved by the Experimental Animal Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine.

2.2.3. Construction and verification of a rat model of IS

Rats underwent middle cerebral artery occlusion to simulate cerebral ischemia. Twenty-four of the 30 rats were randomly selected to prepare the IS; The remaining six rats were used for sham operations. Rats were anesthetized in the supine position. The skin was then cut along the midline of the neck, and the right common carotid artery, external carotid artery, and internal carotid artery, were separated bluntly. Next, we ligated the common carotid artery and the external carotid artery and clamped the internal carotid artery. Then, we inserted a thread plug into the external carotid artery, entered the internal carotid artery, inserted a thread 18–20 mm in length, and then fixed the thread plug. The wire bolt was removed after 2 h. The sham-operation proceeded in the same manner except that the thread plug was not inserted

(Ishrat, et al., 2009). Three rats in the IS model died during the modeling period. None of the rats receiving the sham operation died.

Neurological function score was evaluated 24 h after the operation to observe the neurobehavior of each rat. The lowest total score was 0 (implying that nerve function was normal); A score of 1 referred to an inability to extend the fore paw of the healthy side when the tail was lifted; A score of 2 referred to rotation to the healthy side; A score of 3 referred to an inability to move to the healthy side; And a score of 4 referred to an inability to move independently with a low level of consciousness. Neurological damage was scored 24 h after successful modeling and rats with a neurological deficit score>2 points and<3 points were selected for subsequent experiments.

2.2.4. Screening of IS-induced ED models

Seventeen rats with IS were placed in a quiet and dark observation box for 10 min. Then, Apomorphine (APO) was subcutaneously injected into the neck of each rat at 100 μg/kg. We then recorded each rat in its environment, and noted whether they achieved erection within 30 min (Li & Zheng, 2020). In cases of erection, the penis became swollen or increased in size and protruded from the animal's body. ED was diagnosed if IS rats failed to achieve erection. A total of 12 rats failed to achieve erection and were classified as ISED model rats. These 12 ISED model rats were then randomly divided into two groups: model group (n = 6, hereinafter referred to as the M group) and treatment group (n = 6, hereinafter referred to as the T group). We also included rats from the sham operation group (n = 6, hereinafter referred to as the S group). The intervention methods were as follows. The S group and M group were given deionized water by oral gavage. For the T group, we used deionized water to prepare a 14.9 g/kg solution of HXTLD particle suspension (6 times the amount used for humans). All experimental rats were fed routinely at 10:00 am every day, and drugs were administered to the stomach according at a dose of 1 mL/100 g body weight. The rats were weighed once a week during the experiment and the dose adjusted as appropriate.

2.2.5. Histological analysis

The rats were weighed and anesthetized with sodium pentobarbital (50 mg/kg). The penis was then removed, cleaned, and part of the penis (1 mm in length) was fixed and embedded in paraffin. Thin sections were then prepared (5 μm thick) and stained in hematoxylin-eosin (HE). Then, we evaluated the sections by light microscopy to observe the specimen further and take a camera under an optical microscope equipped with a digital tube (Olympus BX51TF, Tokyo, Japan).

2.2.6. Expression of TNF, eNOS and VEGF protein in cavernous body of penis

Western blotting was used to detect the expression of TNF, eNOS and VEGF proteins in the corpus cavernosum tissue of each rat. Part of the cavernous tissue of each rat was removed and homogenized in ice-cold tissue lysis buffer. The homogenate was then centrifuged at 12 000 rpm for 10 min and the supernatant collected. The BCA protein assay kit was used to determine protein concentration. The concentration of protein was adjusted with RPIA solution and boiled for 5 min. Each sample was then separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Western blotting was then performed with appropriate antibodies at 4 °C overnight with β-actin as an internal control. The following morning, the membrane was washed and incubated with a peroxidase-linked secondary antibody. Finally, we used the ECL detection system to detect positive immune signals.

2.2.7. Expression of TNF, eNOS and VEGF mRNA in cavernous body of penis

Total RNA was extracted from cavernous cells and brain tissue using a commercial kit in accordance with the manufacturer's guidelines. The integrity of the RNA was assessed by agarose gel electrophoresis and purity was verified by spectrophotometry. Following RNA reverse transcription, *in vitro* PCR amplification was performed with a 25 μL reaction system, using β-actin as an internal reference. After pre-denaturation at 95 °C for 5 min, the following cycles are carried out: 94 °C denaturation for 1 min; Annealing at different temperatures for 40 s, 72 °C extension for 1 min, and 30 cycles. This was followed by a final incubation at 72 °C for 10 min to allow full extension. The reaction was carried out in a fluorescent quantitative PCR machine, and all samples were replicated in three parallel wells. At the end of the experiment, we recorded the number of cycles (Ct value) experienced when the fluorescence signal in each reaction tube reached the set threshold. The relative quantification (RQ) method ($RQ = 2^{-\triangle\triangle Ct}$) was used to calculate the multiple of the target gene relative to the reference factor in order to compare differences in gene expression. See Table 1 for specific primer information.

2.2.8. Statistical analysis

All analyses will be performed using SPSS software (version 26.0, Armonk, NY, USA), with a two-sided P value < 0.05 considered significant. Continuous variables will be compared using the t-test or Wilcoxon rank-sum test as appropriate. Since the indicators we collect are all measurement data, the experiment is randomly grouped. After testing, the normality test was P > 0.05 and the variance homogeneity test P > 0.05, so the independent sample t-test was used.

3. Results

3.1. Active ingredients of HXTLD

A total of 764 chemical components were retrieved from the TCMSP database. Using specific criteria (OB ≥ 30% and DL ≥ 0.18), 104 active ingredients were identified; Of these, leech accounted for 13 active ingredients (12.5%), centipede accounted for 5 (4.8%), *Paeoniae Radix Rubra* accounted for 29 (27.9%), *Achyranthis Bidentatae Radix* accounted for 13 (12.5%), *Angelicae*

Table 1
Primer information table.

GeneBank	Primer names	Primer sequences (5′-3′)	Fragment length /bp	Annealing temperature / ℃
β-ACTIN	β-ACTIN 1F	CCTCACTGTCCACCTTCCA	120	66
	β-ACTIN 1R	GGGTGTAAAACGCAGCTCA		65
TNF	TNF 1F	CAGCCAGGAGGGAGAAC	93	63.9
	TNF 1R	GTATGAGAGGGACGGAACC		63.9
VEGF	VEGF 1F	CATCAAGCTCTCTCCTCCA	91	63.2
	VEGF 1R	GGCCTCTTCTTCCACCA		63.5
eNOS	R-ENOS-S	TCACTTACTTCCTGGACATCACTT	254	86
	R-ENOS-A	CTGTTCGCTGGACTCCTCTG		86

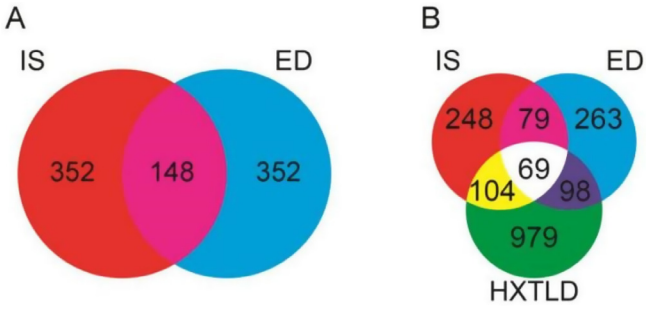

Fig. 1. Venn diagram. Intersection of IS target and ED target (A); intersection of HXTLD target, IS target and ED target (B).

Sinensis Radix accounted for 2 (1.9%), *Vaccariae Semen* accounted for 4 (3.8%), *Curcumae Radix* accounted for 6 (5.8%), *Citri Reticulatae Pericarpium Viride* accounted for 5 (4.8%), *Bupleuri Radix* accounted for 11 (10.6%), *Tribuli Fructus* accounted for 5 (4.8%), *Epimrdii Herba* accounted for 3 (2.9%), and *Morindae Officinalis Radix* accounted for 8 (7.7%).

3.2. Target acquisition

GeneCards and the OMIM database identified 500 IS-related targets and 500 ED-related targets; 148 targets were common to both conditions (Fig. 1A). Analyses involving TCMSP, PubChem, and Swiss Target Prediction identified 1241 targets for HXTLD. Finally, 69 overlapping targets were identified that were common to both HXTLD and ISED (Fig. 1B). The STRING database was then used for PPI analysis.

3.3. Network construction and topology analysis

Cytoscape software was used to construct a 'herbal-active ingredient-target-disease' network (Fig. 2). Next, we performed topology analysis on the network by using the tool "Network analysis" tool. The top four active ingredients (by "degree") were considered to represent the key active ingredients: stigmasterol, kaempferol, quercetin, and β-sitosterol (Table 2). Cytoscape

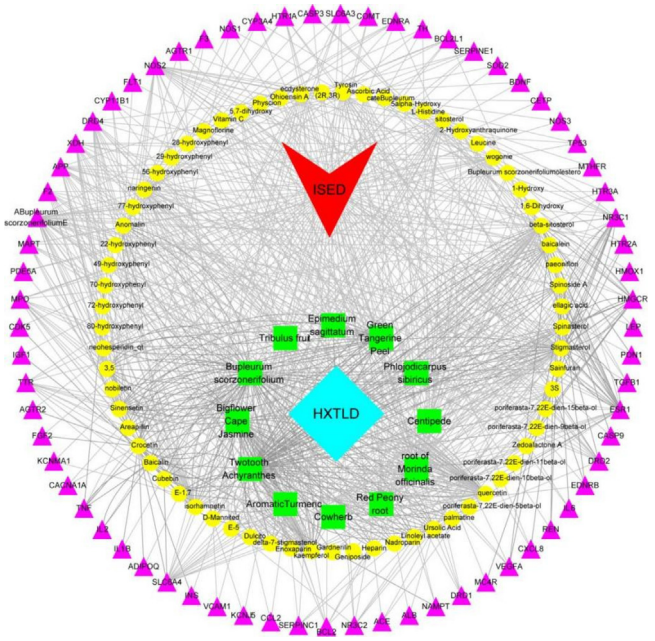

Fig. 2. Network construction of herbal medicine-active ingredient-target-disease. The red node represents ISED, the blue diamond represents HXTLD, the green square represents herbal medicine, the yellow circle represents the active ingredient, and the pink triangle represents the target.

Table 2
Specific information of four key active ingredients.

Molecular names	Molecular ID	Degree
Stigmasterol	MOL000449	52
Kaempferol	MOL000422	48
Quercetin	MOL000098	48
Beta-sitosterol	MOL008583	46

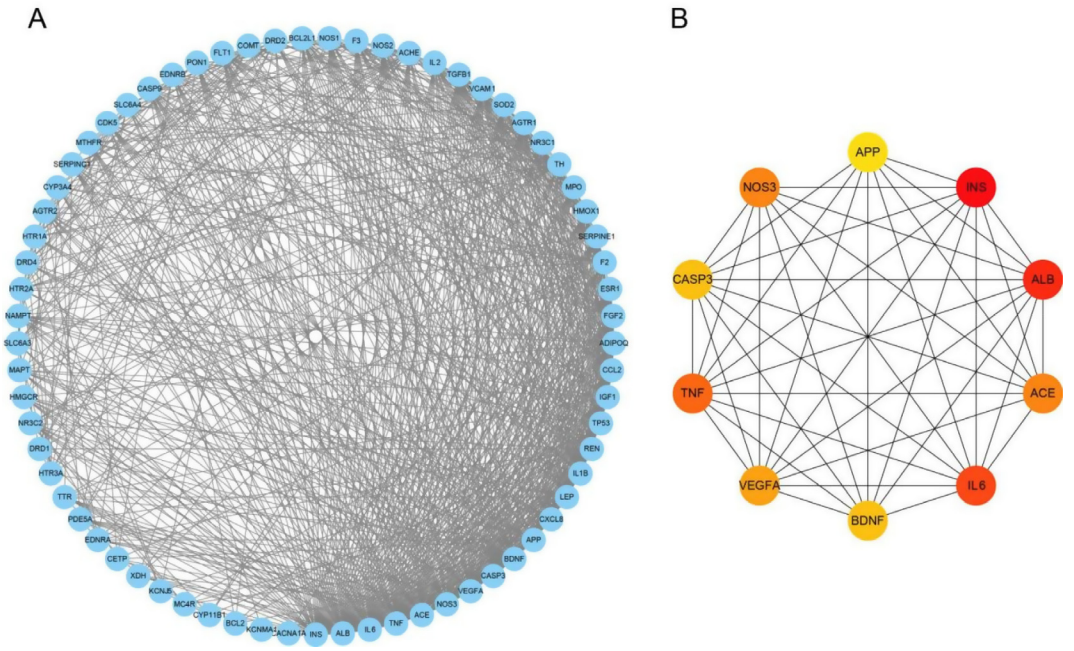

Fig. 3. PPI network diagram. (A) PPI network established by Cytoscape (3.7.1), the red node is the first five key nodes processed by cytohubba, and the size of the node is proportional to the "degree" of the node; (B) PPI network processed by the plug-in (cytohubba), the color of the node is proportional to the "degree".

Table 3
Specific information of top 10 key targets.

Rank	Names	Degrees
1	INS	58
2	ALB	52
3	IL6	48
4	TNF	45
5	ACE	44
5	NOS3	44
7	VEGFA	43
8	CASP3	40
8	BDNF	40
10	APP	39

software was also used to construct a PPI network (Fig. 3A). By using a Cytoscape software plug-in (cytohubba), we were able to determine the top 10 targets (by "degree"): Insulin (INS), albumin (ALB), interleukin 6 (IL6), TNF, angiotensin I converting enzyme (ACE), eNOS, VEGF, Caspase 3 (CASP3), brain derived neurotrophic factor (BDNF), amyloid beta precursor protein (APP) (Table 3 and Fig. 3B).

3.4. GO and KEGG enrichment analysis

Enrichment analysis of the 69 targets using DAVID v6.8 software showed that HXTLD involves 137 cell biological processes (BP), 20 cell components (CC), 29 molecular functions (MF), and 60 signal pathways. According to *P* values, the top 5 biological processes and pathways were identified (Fig. 4).

3.5. Analysis of number of erections in each group of rats

The mean number of erections in group S within the experimental time period was 3.33 ± 0.94, the number of erections in group M was 0. The number of erections in rats from group M was significantly lower than that in group S ($P < 0.05$). The number of erections in group T was 1.67 ± 0.47; This was significantly higher than rats in the M group ($P < 0.05$).

3.6. Tissue structure of cavernous body of penis

HE staining showed that there were cavernous trabeculae and evenly distributed sinuses in the corpus cavernosum of rats from S group, along with some red blood cells in the sinus cavity. The trabecular sinus contained an abundance of smooth muscle, collagen fibers, and some small blood vessels; there was no proliferation of the interstitial tissue. However, in the corpus cavernosum of the M group, the sinusoidal distribution was disordered and the density of endothelial cells and smooth muscle cells was significantly reduced. Compared with the M group, after 8 weeks of drug intervention, the sinusoidal distribution in rats from the T group was relatively regular, the endothelial cell density had increased, and red blood cells were visible in some sinusoidal spaces (Fig. 5).

3.7. Expression of TNF, eNOS and VEGF protein in cavernous body of penis

The levels of TNF protein in the penile tissue of rats from the M group were significantly higher than those in the S group ($P < 0.01$). The levels of TNF protein in rats from group T were significantly lower than those in group M ($P < 0.05$). The levels of VEGF protein in penile tissue in rats from group M were significantly higher than those in the S group ($P < 0.01$). The levels of VEGF protein in rats from the T group were significantly lower than those in the M group ($P < 0.05$). The levels of eNOS protein level in the penile tissue of rats from the M group were significantly lower ($P < 0.01$) than those in the S group. The levels of eNOS protein in rats from the T group were significantly higher than those in the M group ($P < 0.05$; Fig. 6).

3.8. Expression of TNF, eNOS, VEGF mRNA in cavernous body of penis

The levels of TNF mRNA in the penile tissue of rats from the M group were significantly higher than those in the S group ($P < 0.01$). The levels of TNF mRNA in the T group were significantly lower than those of the M group ($P < 0.05$). The levels of VEGF mRNA in the penile tissue of rats in the M group were significantly higher than those in the S group ($P < 0.01$). The levels of VEGF mRNA in the

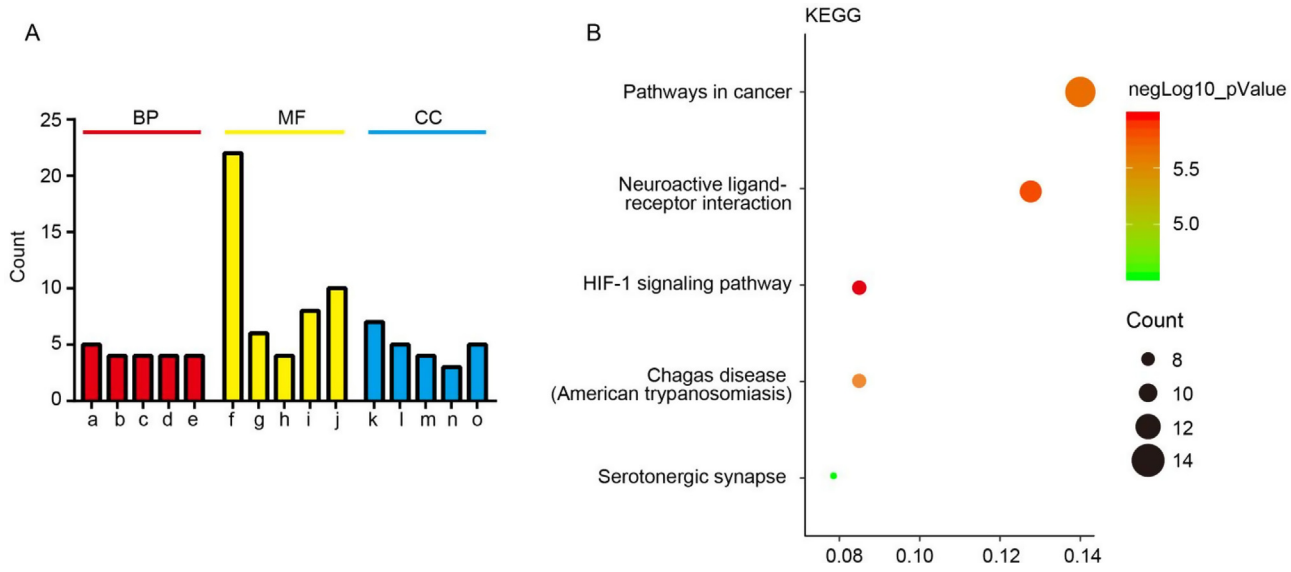

Fig. 4. GO and KEGG enrichment analysis. (A) GO item analysis: the red bar represents BP, the yellow bar represents CC, and the blue bar represents MF (a: response to DNA damage b: synaptic transmission, dopaminergic c: neuron apoptotic process d: lipopolysaccharide-mediated signaling pathway e: protein kinase B signaling f: extracellular space g: axon h: blood microparticle i: extracellular region j: integral component of plasma membrane k: growth factor activity l:drug binding m: dopamine binding n: nitric-oxide synthase activity o: flavin adenine dinucleotide binding). (B) KEGG pathway enrichment: X-axis represents rich factor, Y-axis represents name, node size is related to count, and node color is related to *P* value.

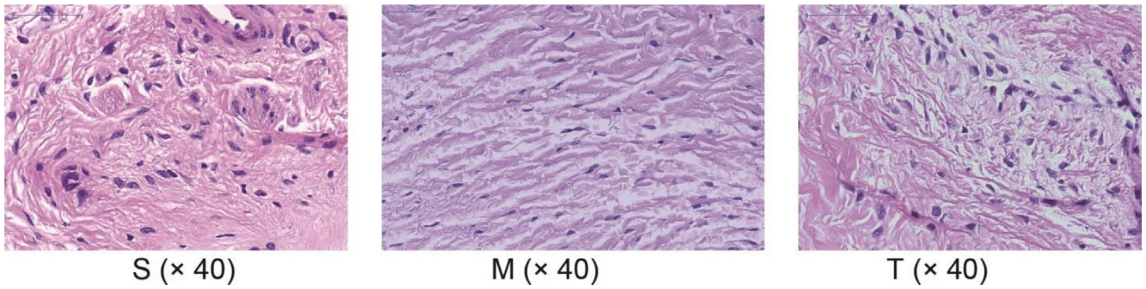

Fig. 5. HE staining results of cavernous body of penis. The trabecular sponge and sinuses were evenly distributed in the cavernous body in the S group of rats. The density of endothelial cells and smooth muscle cells in group M decreased significantly. The sinusoidal distribution of rats in the T group was more regular, the density of endothelial cells increased, and red blood cells were visible in some sinusoidal spaces.

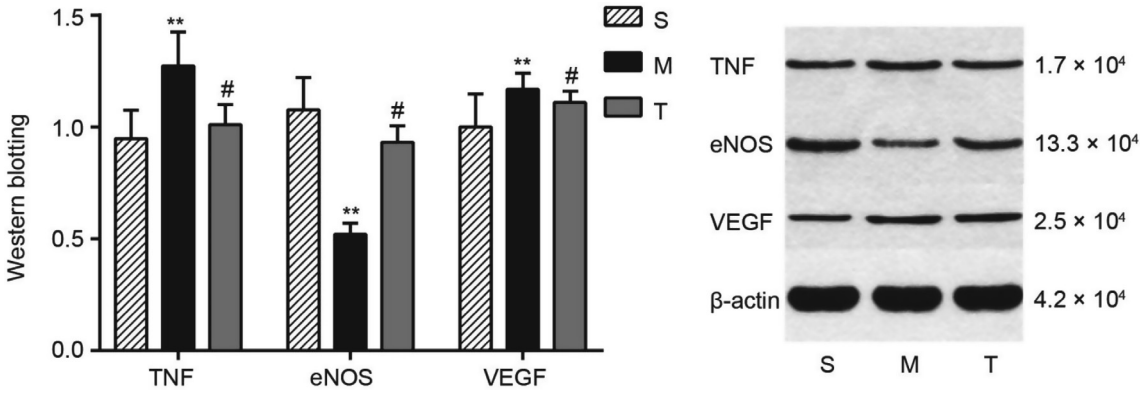

Fig. 6. Expression of TNF, eNOS and VEGF protein in cavernous body of penis by Western blotting analysis. $^{**}P < 0.01$ compared with S group, $^{\#}P < 0.05$ compared with M group.

T group were significantly lower than those in the M group ($P < 0.05$). The levels of eNOS mRNA in the penile tissue of rats in the M group were significantly lower than those in the S group ($P < 0.01$). The levels of eNOS mRNA in rats from the T group were significantly higher than those in the M group ($P < 0.05$; Fig. 7).

4. Discussion

Previous studies have shown that ED and many cardiovascular diseases have common risk factors. Consequently, the risk factors for cardiovascular disease are significantly associated with ED, including age, body mass index, cholesterol, triglycerides, smoking, and hypertension (Guo, Wang & Luo, 2020; Zhou, et al., 2020). In addition, diabetes is also known to increase the risk of both diseases. Endothelial cell dysfunction and arteriosclerosis are also common features of both E D and cardiovascular disease. Autonomic hyperfunction and changes in hormone levels may also be involved and likely to involve complex mechanisms. The close correlation between cardiovascular disease and cerebrovascular disease, in terms of pathogenicity, pathophysiology, progression, and prognosis, has been unanimously recognized by the medical community. Therefore, it is reasonable to believe that ED and cerebrovascular disease are similarly related (Kimura, et al., 2013; Pase, et al., 2012). The current results support these earlier findings. Since the mechanism underlying ED in patients with IS remains unclear, we combined network pharmacological prediction and animal experimentation to investigate the mechanistic associations between IS and ED.

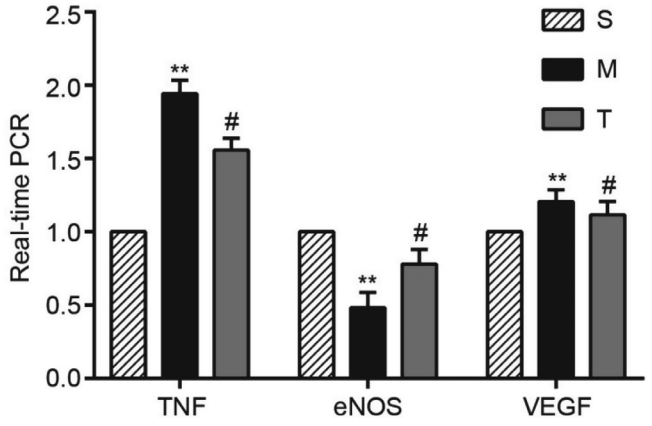

Fig. 7. Expression of TNF, eNOS and VEGF mRNA in cavernous body of penis by Real-time PCR analysis. $^{**}P < 0.01$ compared with S group; $^{\#}P < 0.05$ compared with M group.

Network pharmacology studies identified several key targets of HXTLD for ISED: INS, ALB, IL6, TNF, ACE, eNOS, VEGF, CASP3, BDNF, and APP. Based on extensive literature review and preliminary basis, we selected TNF, eNOS and VEGF for testing.

Experiments showed that IS caused a significant reduction in erectile function in rats and caused damage to the tissue structure of the spongy endothelial cells and smooth muscle cells in the penis. This appears to be the direct cause of the decline in erectile function. We also found that HXTLD significantly improved these pathological changes in a rat model of ISED. HXTLD also inhibited the expression of TNF and VEGF protein in rat penile tissue, and promoted the expression of eNOS protein in penile tissue. Studies have reported that cerebral ischemia can cause the expression of TNF mRNA in the brain. Ischemia increases the permeability of

the blood–brain barrier and allows mononuclear macrophages in the peripheral blood to enter the brain tissue, thus promoting the increase of TNF and other cytokines. Many TNF proteins can directly inhibit the expression of eNOS mRNA and reduce eNOS activity (Hu, et al., 2016). A series of signal pathways mediated by TNF are known to initiate and accelerate arteriosclerosis, thrombosis, vascular remodeling, and the activation of endothelial cell apoptosis pathways, thus increasing the risk of ED (Jiang, et al., 2017). Neuronal nitric oxide synthase (nNOS) is considered to be an important non-adrenergic non-cholinergic (NANC) neurotransmitter that relaxes penile tissue. Studies have found that animals in which TNF-α has been knocked out show increased expression levels of NOS in the cavernous tissue. This indicates that TNF-α inhibits the expression of NOS in the cavernous tissue, thereby reducing the main messenger NO that induces penile erection and causes ED (Leungwattanakij, et al., 2003). Therefore, the results show that TNF is also one of the most important targets of HXTLD and can result in ISED. The experimental results are consistent with the results predicted by network pharmacology. Some scholars have reported that eNOS is a key gene associated with ED and acts via the PI3K/AKT pathway (Adilijiang, et al., 2008); The current results are consistent with these previous findings. We found that the expression of eNOS protein and mRNA in the ISED model group was significantly inhibited, but could be improved by treatment with HXTLD. This indicated that eNOS is one of the mechanisms underlying the effects of HXTLD in the treatment of ISED. Under pathological conditions, the high expression levels of VEGF and its receptor can cause structural changes in the extravascular matrix and interstitial edema (Zhao, et al., 2018). The overexpression of VEGF can increase vascular permeability, aggravate the inflammatory response, and further aggravate the occurrence of ED (Urios, et al., 2019).

Therefore, we used network pharmacology and *in vivo* experiments and hypothesize that the possible mechanism linking IS to ED is atherosclerosis. The process of atherosclerosis plays an important role in the process of cerebral ischemia–reperfusion injury (Lavallée, et al., 2002). TNF, as an inflammatory factor, plays a vital role in the formation and rupture of atherosclerotic plaques (Hahn & Hill, 2015). An excess of TNF inhibits the expression of eNOS, thus leading to a reduction in NO and an inhibition of erectile function. In addition, an excess of TNF can promote an increase of related factors that activate the coagulation system, accelerate the coagulation process (Page, Bester & Pretorius, 2018), cause thrombosis, and further aggravate ED. After stroke, the function of the vascular endothelium changes; this leads to a pathological increase in the levels of VEGF (Zhang, et al., 2017). Furthermore, an excess of VEGF will increase vascular permeability, aggravate inflammation, and reperfusion injury; his is not conducive to stroke.

5. Conclusion

In summary, HXTLD significantly improved the erectile function of rats with ED caused by IS, and exerted a regulatory effect on the levels of TNF, eNOS and VEGF protein and mRNA. This is likely to be a key mechanism to consider when attempting to improve erectile function. This research innovatively combined network pharmacology with *in vivo* research, and identified the mechanisms that might allow us to develop new and innovative treatments for ISED. However, this study involves an experimental study that was based on network pharmacology and bioinformatics predictions. The prediction results did not involve sex hormone-related indicator; Consequently, these were not tested during the experiment. However, considering the hypoxia mechanism associated with IS can also lead to endocrine dysfunction, and that erectile function is closely related to hormone level, this might represent a limitation to our findings. Follow-up experiments should involve the detection of key hormone levels so that we can provide more rigorous guidance for the clinical treatment of ISED.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adilijiang, Y., Kesseljiang, D., Li, X., et al. (2008). The role of nitric oxide synthase in diabetic erectile dysfunction in rats. *Journal of Xinjiang Medical University, 08*, 937–939.
Guo, P., Wang, J., & Luo, J. (2020). The effect of soothing liver, relieving depression and promoting blood circulation on homocysteine in patients with post-stroke depression. *Yunnan Journal of Traditional Chinese Medicine and Materia Medica, 41*(06), 34–35.
Hu, H., Shen, C., Xu, S., et al. (2016). Clinical observation of the correlation between erectile dysfunction caused by OSAHS and serum NO and NOS. *Chinese Sexual Sciences, 25*(01), 5–7.
Hahn, C., & Hill, M. D. (2015). Early anti-coagulation after ischemic stroke due to atrial fibrillation is safe and prevents recurrent stroke. *Canadian Journal of Neurological Sciences, 42*(2), 92–95.
Hua, Y. L., Ma, Q., Yuan, Z. W., et al. (2019). A novel approach based on metabolomics coupled with network pharmacology to explain the effect mechanisms of Danggui Buxue Tang in anaemia. *Chinese Journal of Natural Medicines, 17*(4), 275–290.
Ishrat, T., Sayeed, I., Atif, F., et al. (2009). Effects of progesterone administration on infarct volume and functional deficits following permanent focal cerebral ischemia in rats. *Brain Research, 1257*, 94–101.
Jiang, B., Yin, S., Chen, G., et al. (2017). Expression and significance of tumor necrosis factor in the serum of patients with erectile dysfunction. *Chinese Medical Innovation, 14*(3), 5–8.
Kimura, M., Shimura, S., Tai, T., et al. (2013). A web-based survey of erection hardness score and its relationship to aging, sexual behavior, confidence, and risk factors in Japan. *Sexual Medicine, 1*(2), 76–86.
Lin, A. X., Chan, G., Hu, Y., et al. (2018). Internationalization of traditional Chinese medicine: Current international market, internationalization challenges and prospective suggestions. *Chinese Medicine, 13*(1), 9.
Leungwattanakij, S., Bivalacqua, T. J., Usta, M. F., et al. (2003). Cavernous neurotomy causes hypoxia and fibrosis in rat corpus cavernosum. *Journal of Andrology, 24*(2), 239–245.
Li, X., 2019. Clinical and experimental study of Huoxue Tongluo Qiwei Decoction on diabetic erectile dysfunction. Beijing University of Chinese Medicine, 2019.
Li, L., & Zheng, M. (2020). Application of activating blood and removing blood stasis drugs in the treatment of erectile dysfunction. *China Prescription Drugs, 18*(05), 14–16.
Lavallée, P., Perchaud, V., Gautier-Bertrand, M., et al. (2002). Association between influenza vaccination and reduced risk of brain infarction. *Stroke, 33*(2), 513–518.
Lee, Y. W., Chen, T. L., & Shih, Y. R. V. (2014). Adjunctive traditional Chinese medicine therapy improves survival in patients with advanced breast cancer: A population-based study. *Cancer, 120*(9), 1338–1344.
Liu, D., Wang, Q., Wei, B., et al. (2019). Network pharmacology study of Kunming mountain crabapple-*Spatholobus spatholobi* medicine on the treatment of rheumatoid arthritis. *China Pharmacy, 30*(19), 2639–2644.
Lisa, W., & Friederike, (2019). Network pharmacology for antiepileptogenesis: Tolerability and neuroprotective effects of novel multitargeted combination treatments in nonepileptic vs. post-status epilepticus mice. *Epilepsy Research, 151*, 48–66.
Liu, Q., & Dai, J. (2017). Observation on the clinical efficacy of self-made Huoxue Yiqi Recipe on patients with acute ischemic stroke of Qi deficiency and blood stasis type. *Journal of Hubei University of Traditional Chinese Medicine, 19*(3), 50–53.
Ma, J., 2017. Epidemiological study of erectile function and sexual life in 248 male patients with ischemic stroke. Beijing University of Chinese Medicine, 2017.
Pase, M. P., Grima, N. A., Stough, C. K., et al. (2012). Cardiovascular disease risk and cerebral blood flow velocity. *Stroke, 43*(10), 2803–2805.
Page, M. J., Bester, J., & Pretorius, E. (2018). The inflammatory effects of TNF-α and complement component 3 on coagulation. *Scientific Reports, 8*(1), 1812.
Shindel, A. W. (2012). Erectile dysfunction and risk of cardiovascular disease: Meta-analysis of prospective cohort studies. *Yearbook of Urology, 2012*, 114–115.
Tsertsvadze, A., Fink, H. A., Yazdi, F., Macdonald, R., Bella, A. J., Ansari, M. T., et al. (2009). Oral phosphodiesterase-5 inhibitors and hormonal treatments for

erectile dysfunction: A systematic review and meta-analysis. *Yearbook of Urology, 2010*(9), 146–147.
Urios, A., Ordoño, F., García-García, R., et al. (2019). Tadalafil treatment improves inflammation, cognitive function, and mismatch negativity of patients with low urinary tract symptoms and erectile dysfunction. *Scientific Reports, 9*(1), 17119.
Wang, D., Li, R., Wei, S., et al. (2019). Metabolomics combined with network pharmacology exploration reveals the modulatory properties of *Astragali Radix* extract in the treatment of liver fibrosis. *Chinese Medicine, 14*, 30.
Wang, J. S., Li, X., Chen, Z. L., et al. (2021). Effect of leech-centipede medicine on improving erectile function in DIED rats via PKC signalling pathway-related molecules. *Journal of Ethnopharmacology, 267* 113463.
Xie, D. (2016). Clinical observation on the treatment of male erectile dysfunction with acupuncture and medicine. *Journal of Hubei University of Traditional Chinese Medicine, 18*(02), 29–31.
Yuan, J., Zhang, R., & Yang, Z. (2013). PDE5, Erectile Dysfunction. *European Urology, 63*(5), 902–912.
Zhang, S., Xiang, Y., Yang, Q., et al. (2020). Network pharmacological analysis of Xinguankangfu Granules on the protective mechanism of pulmonary fibrosis after the new coronavirus pneumonia. *Journal of Hubei University of Traditional Chinese Medicine, 22*(05), 16–21.
Zhou, S., Liu, F., Deng, Y., Li, B., Zeng, C., Wu, Z., et al. (2020). Retrospective study of Qixiantongluo prescription in the treatment of cerebral infarction recovery and sequelae of kidney deficiency and blood stasis syndrome. *Hunan Journal of University of Chinese Medicine, 40*(10), 1283–1289.
Zhao, X., Yu, F., Huang, X., et al. (2018). Azithromycin influences airway remodeling in asthma via the PI3K/Akt/MTOR/HIF-1α/VEGF pathway. *J Journal of Biological Regulators and Homeostatic Agents, 32*(5), 1079–1088.
Zhang, H. T., Zhang, P., Gao, Y., et al. (2017). Early VEGF inhibition attenuates blood-brain barrier disruption in ischemic rat brains by regulating the expression of MMPs. *Molecular Medicine Reports, 15*(1), 57–64.