Original Article

Integrated Network Pharmacology and Antioxidant Activity-Guided Screen System to Exploring Antioxidants and Quality Markers of Shunaoxin Pills against Chronic Cerebral Ischemia

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Abstract

Objective: The main objective of the study is to screen the quality markers (Q-markers) for relieving oxidative stress damage and against chronic cerebral ischemia in Shunaoxin pills (SNX). **Methods:** The benefit effect of SNX was evaluated by a rat chronic cerebral ischemia model. The main ingredients of SNX were identified by ultra-performance liquid chromatography-quadrupole time-of-flight, whereas its core targets and pathways around antioxidative stress were predicted by PharmMapper and kyoto encyclopedia of genes and genomes (KEGG) analysis. Moreover, the antioxidants were screened by high-performance liquid chromatography with postcolumn derivatization system and then representative ingredients were verified by cell experiments. **Results:** SNX could increase expression of catalase and superoxide dismutase (SOD) as well as antagonize oxidative damage in the brain. The effects may be related to three types of antioxidant pathways, including nitrogen metabolism, arachidonic acid metabolism, and the cyclic guanosine monophosphate-dependent protein kinase (cGMP-PKG) signaling pathway by multiple active components regulate targets. Among them, ferulic acid and ligustilide were shown the key scavenging ability for reactive oxygen free radicals and significantly increased the contents of nitric oxide (NO), NO synthase, and SOD as well as decreased malonaldehyde. **Conclusion:** The oxidation resistances of biological and chemical processes in SNX to protect against cerebral oxidative stress injury were preliminary revealed by an integrated network pharmacology and antioxidant activity-guided screen system. Ferulic acid and ligustilide played a major antioxidant role that could be used as Q-markers to control the quality of SNX.

Keywords: Antioxidant, chronic cerebral ischemia, network pharmacology, Q-markers, Shunaoxin pills

Introduction

Chronic cerebral ischemia is the leading cause of adult disability from Alzheimer's disease and vascular dementia, which are accepted as the most common forms of dementia.[1,2] In recent years, oxidative stress has been shown to play an important role in ischemic brain injury. The ischemic areas show activation of free-radical processes and the generation of reactive oxygen species, leading to lipid peroxidation, which eventually causes cell injury.[3] Superoxide dismutase (SOD) and catalase (CAT) are known to effectively remove oxygen free radicals and peroxides through chronic cerebral ischemic injury.[4] Numerous antioxidants, such as ginkgo and oxiracetam, have shown neuroprotective

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effects in ischemia cerebral injury with satisfactory clinical results.[5,6] Shunaoxin pills (SNX) are composed of Chuanxiong Rhizoma, which contains the roots of *Ligusticum chuanxiong* Hort. and *Angelica sinensis* Radix as well as the root of *A. sinensis* (Oliv.) Diels, which are treatments from traditional Chinese medicine (TCM) that are known to improve cerebral microcirculation and oxygen delivery as well as reinforce learning and memory.[7,8] A clinical study showed that SNX had an obvious preventative effect against ischemic stroke.[9] However, the mechanism and effect of the constituents of SNX are not clear. Chuanxiong Rhizoma and Angelica have been found to contain many active components, such as Z-ligustilide, ligustrazine, and ferulic acid, which have significant antioxidant pharmacological activities.[10-12] Nevertheless, it is not clear which ingredients in SNX have some comprehensive beneficial effects.

As is known to all, the quality of TCM forms the foundation of its clinical efficacy. How to effectively control TCM quality remains a challenge. In 2016, a novel concept of the TCM quality marker (Q-marker) was proposed by Academician Liu.[13] Focusing on the concept, Chinese scholars have been conducting Q-marker research about its discovery and application and gradually formed a new quality research model.[14,15] In this article, we propose a new research strategy, which integrated network pharmacology and activity-guided screen system to exploring Q-markers in SNX. After evaluating the protective effect of SNX in the rat model of chronic cerebral ischemia, the main chemical components in SNX were identified by ultra-performance liquid chromatography-quadrupole time-of-flight (UPLC/Q-TOF). Then, their potential targets focused on oxidative stress pathways were determined through network pharmacology methods. At the same time, the antioxidants in SNX were screened by a novel high-performance liquid chromatography (HPLC) postcolumn derivatization system and then the key ingredients were recommended as Q-markers and verified through an oxidative damage model in endothelial cell level.

Methods

Sample preparation

About 100 mg of SNX extract (Tianjin Zhongxin Pharmaceutical Group Co., Ltd., Tianjin, China) and 1 mg of ferulic acid and ligustilide (Tianjin YIFANG S and T Co., Ltd., Tianjin, China) were dissolved in 10 mL of methanol for UPLC analysis. Nicergoline tablets (Kunshan KRRP Pharmaceutical Co., Ltd., Jiangsu, China) and SNX were diluted with physiological saline for animal experiments.

Chronic cerebral ischemia test in rats

Sprague-Dawley (SD) male rats, which weighed 250–300 g, were purchased from the Beijing Experimental Animal Center of Military Medical Sciences (Beijing, China). The animals were raised in a 12 h automatic light/dark cycle in a standard animal room with the temperature maintained at 23°C–26°C. Rats had free access to water and food until the day of the operation.

A chronic cerebral ischemia model was established by ligating the bilateral common carotid artery.[16] After the procedure was finished, rats were treated with normal saline, nicergoline, or different doses of SNX once a day for 2 months using oral gavage. Thirty minutes after the last treatment, the brain tissues were collected and divided into two parts. Half of the samples were preserved in formalin for HE staining, and the other half were used to detect the SOD and CAT contents with ELISA kits (Xitang Company, Shanghai, China).

Ultra-performance liquid chromatography-quadrupole time-of-flight analysis

The sample was passed through a 0.22 μm microporous membrane and analyzed by an UPLC/Q-TOF system. A Waters Acquity UPLC System (Waters Co., Milford, America) was equipped with a photodiode array detector, which achieved in a range of 190–400 nm. The system was controlled using Mass Lynx V4.1 software (Waters Co.). An Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters Co.) was used for the separations. The injection volume was 2.0 μL with a gradient elution of acetonitrile (A) and a 0.1% formic acid aqueous solution (B) using the following protocol: 2% to 27% A at 0–8 min, 27% to 35% A at 8–15 min, 35% to 50% A at 15–20 min, 50% to 65% A at 20–27 min, 65% to 73% A at 27–32 min, 73% to 100% A at 32–35 min, and 100% to 2% A at 35–38 min, and then, the system was maintained at 2% A from 38 to 40 min. The flow rate was 0.40 mL/min, and the column temperature was maintained at 30°C.

Tandem mass spectrometry (MS/MS) was performed on a Waters UPLC/Q-TOF Premier with an electrospray ionization (ESI) system (Waters MS Technologies, Manchester, UK). The ESI-MS spectra were acquired in both the negative and positive ion voltage modes. The ion source temperature was 110°C. The capillary voltage was set to 2.5 kV for the negative mode and 3.0 kV for the positive mode. The sample cone voltage was set to 30 V. High-purity nitrogen was used as the nebulization agent in the negative mode and auxiliary gases were used in the positive mode. The nitrogen gas flow rate was 600 L/h and temperature was 350°C, and the cone gas was set to 50 L/h. The acquisition rate was 0.1 s with a 0.02 s interscan delay. The mass detection range was 100–1500 Da. Leucine enkephalinamide acetate (200 μg/L) was used as the lock mass ($[M-H]^-$ =553.2775, $[M+H]^+$ =555.2931).

Targets and pathway prediction

Using Lipinski's rule of five and topological polar surface area (TPSA) (http://www.molinspiration.com/cgi-bin/properties), the absorbability of the compounds identified in SNX was calculated. The compounds that met the rules and had TPSAs ≤140 as well as rotatable bonds ≤10 were deemed to be good orally absorbable compounds. The compounds that met both criteria were considered to be orally available compounds. Next, the three-dimensional structures of the identified compounds that were absorbed by the body were entered into the PharmMapper database (http://59.78.96.61/phammapper) for target prediction. The pathways were

determined through KEGG pathway analysis (http://bioinfo.capitalbio.com). Protein interactions were analyzed using String 10.5 (http://www.string-db. org/).

High-performance liquid chromatography -ultraviolet-fluorescence detectors analysis system

All analyses were carried out on a liquid chromatography system (LC-20AD, Shimadzu, Japan) equipped with ultraviolet (UV) (SPD-20A, Shimadzu, Japan) and fluorescence (FLD) (RF-20A, Shimadzu, Japan) detectors. The measurements were conducted with two solvent delivery pumps (LC-20 AT, Shimadzu, Japan) and two derivatization solvent delivery pumps (LC-10 AT, Shimadzu, Japan). Separation was achieved on a water symmetry C18 column (250 mm × 4.6 mm i.d., 5 μm, Phenomenex, California, USA). The injection volume was 20 μL and a gradient elution of a 0.1% formic acid aqueous solution (A) and acetonitrile (B) was performed as follows: 5% to 27% B at 0–15 min, 27% to 33% B at 15–30 min, 33% to 54% B at 30–40 min, 54% to 69% B at 40–50 min, 69% to 71% B at 50–55 min, 71% to 80% B at 55–65 min, 80% to 85% B at 65–70 min, 85% to 90% B at 70–75 min, 90% to 100% B at 75–80 min, and 100% to 5% B at 80–85 min, and then, the system was maintained at 5% B from 85 to 90 min. The flow rate was set at 1 mL/min, and the column temperature was maintained at 35°C in a column oven (CTO-10AS, Scienhome, Tianjin, China). The separated compounds were first detected at 260 nm using a UV detector and then used in the following derivation process. The postcolumn derivatization was based on a simultaneous supply of a 3% H_2O_2 solution and derivatization reagent that contained 8×10^{-6} mol/L hemin (Meryer Chemical Technology Co., Ltd., Shanghai, China) and 8×10^{-5} mol/L 4-hydroxyphenylacetic acid (PHPAA) (Macklin Biochemical Technology Co., Ltd., Shanghai, China) dissolved in an $NH_4Cl/NH_3.H_2O$ buffer solution (pH 10.5). The flow rates were set at 0.6 mL/min and 0.2 mL/min. The derivatization reaction was maintained throughout the length of a polyether ether ketone tube (0.18 mm × 3 m) at 25°C. Moreover, the elution was detected by FLD detector, the excitation wavelength was 315 nm, and the emission wavelength was 400 nm.

Cell validation

Mouse brain cerebral cortex cell line, B End. 3 cells (American Type Culture Collection, USA) were cultured in Dulbecco minimum essential medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco, USA) in a 37°C humidified incubator with a 5% CO_2 supply. After the cells reached 80%–90% confluence, they were stimulated with 100 μmol/L H_2O_2 for 30 min, except for cells in the control group. Next, the cells were treated with ferulic acid, ligustilide (Tianjin YIFANG S and T, Tianjin, China), and Vitamin C (Bayer, Germany) for 6 h, and then, the contents of nitric oxide (NO) and activities of NO synthase (NOS), malonaldehyde (MDA), and SOD were detected according to the kit instructions (Nanjing Jiancheng Bio, Shanghai, China).

Statistical analysis

All data are expressed as the means ± standard deviation, statistical comparisons were conducted among groups with the *t*-test. All statistical analyses were performed using SPSS version 18.0 statistical analysis software (SPSS, Inc., Chicago USA). Statistical significance was set at $P < 0.05$.

Results and Discussion

The protection effects of Shunaoxin pills in Chronic cerebral ischemia

Usually, white matter occupies approximately 50% of the area in the brain tissue and is associated with intelligence activities, such as memory, behavior, and emotions, with fibers traveling through the peripheral edges around the ventricles.[17,18] When white matter is damaged by oxidation, the fiber connections through a site are interrupted, resulting in varying degrees of cognitive impairment.[19] As shown in Figure 1, in control group brain tissues, the number and morphology of nerve cells were normal, and there was no ischemia or necrosis. The model group showed that the brain tissue was sparser, neuronal perikarya exhibited the characteristic morphological features of ischemic damage in the parietal cortex, and subcortical tissue appeared to be loose and showed signs of edema. However, for the positive group with nicergoline (Positive, 2 mg/kg) and for the SNX treatment groups (SNX[H], 20 mg/kg, SNX[M], 10 mg/kg, SNX[L], 5 mg/kg), the nerve cells were arrayed normally along the axon and the white matter was not loose in the corpus callosum or in the subcortical and cerebral ventricles. This outcome suggested that SNX improved the ischemic injury after the operation.

To further determine the benefits of SNX, we evaluated the activities of SOD and CAT, which are two important antioxidant indices in rat serum of rats. As shown in Figure 2, the chronic cerebral ischemia model resulted in a significant decrease in CAT and SOD activities compared to the control group ($P < 0.001$, $P < 0.05$). The SNX high-dose treatment markedly reduced these changes in chronic cerebral ischemia as well as increased the CAT and SOD levels ($P < 0.01$, $P < 0.05$). It was reported that chronic cerebral ischemia due to a large amount of oxygen free radicals consumes excessive

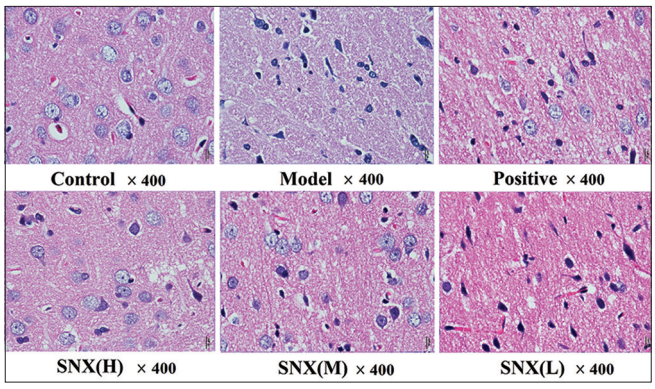

Figure 1: The protective effects of Shunaoxin pills in white matter chronic cerebral ischemia rats

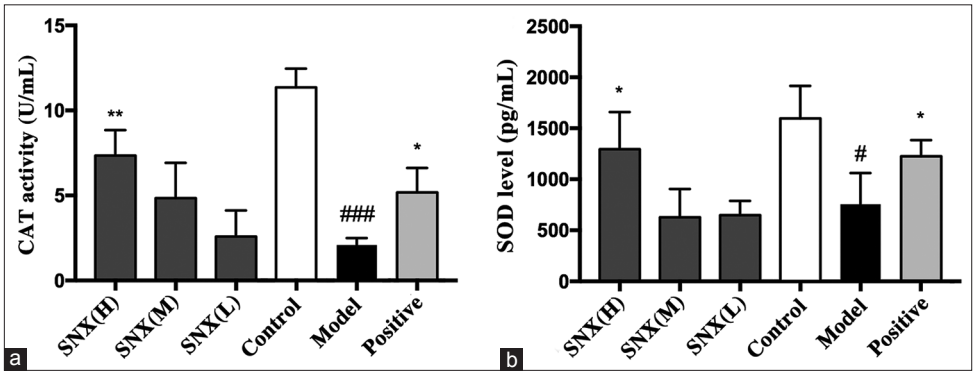

Figure 2: The effects of SNX on regulating CAT and SOD expression in the serum of rats with chronic cerebral ischemia. The SNX could significantly increased CAT activity (a), The SNX could significantly increased SOD level (b). Each bar represents the mean ± SD ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the model group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, the model group compared to the control group

CAT and SOD, resulting in reduced CAT and SOD levels in brain tissue as well as causing damage to the structure and function of the brain biofilm.[20,21] The results indicated that SNX may play an important role through antioxidant effects.

Identification of potential active compounds in Shunaoxin pills

To profile the holistic compounds in SNX, UPLC/Q-TOF assay was carried out. Detailed results about separation and identification of MS/MS information were shown in Figure 3 and Table 1. Overall, 18 main chemical constituents from SNX were identified and mainly classified into five chemical structure types, including lactones (diligustilide, 4-OH-3-butylphthalide, cnidilide, senkyunolide I, ligustilide, neoligustilide, bergapten, and phthalic anhydride), phenolic acids (vanillic acid, protocatechuic acid, ferulic acid, caffeic acid, guaiacol, isoeugenol, and anisic acid), alkaloids (ligustrazine), terpenes (spathulenol), and anthraquinones (chrysophanol). However, some of these components may not be absorbed in the human body, so we calculated the absorption properties of these components. As shown in Table 1, 17 components were proposed to be matched Lipinski's rule of five.

The targets and pathway predictions of the potential active components in Shunaoxin pills

Using virtual calculations in PharmMapper, 17 ingredients that have absorption properties were predicted to regulate a total of 47 targets (PTGS2, PRKACA, PTGS1, CDK2, CCNA2, ESR2, ESR1, CA1, HBA2, CA2, OPRM1, OPRK1, PDE4D, OPRD1, SCN5A, PGR, HSD17B1, AR, GLTP, LCN2, ACHE, NR3C2, ADRB2, ADRB1, PRSS1, POLK, ADRA2A, HTR2A, DRD2, DRD1, NCOA1, CHRM1, NOS2, PLA2G1B, ADRA1A, PDE4B, CA4, NOS3, IFNG, GABBR1, ABCC8, GABRA1, GABRA5, HRH1, ADRA2B, DHFRP1, and TUBA1A). As shown in Table 1, we analyzed the 15 main components of SNX, which regulate ten oxidative stress-related targets with a norm fit value ≥0.3 by annotation. These targets were part of cGMP-PKG signaling pathway, arachidonic acid metabolism, and nitrogen metabolism signaling pathways chiefly.

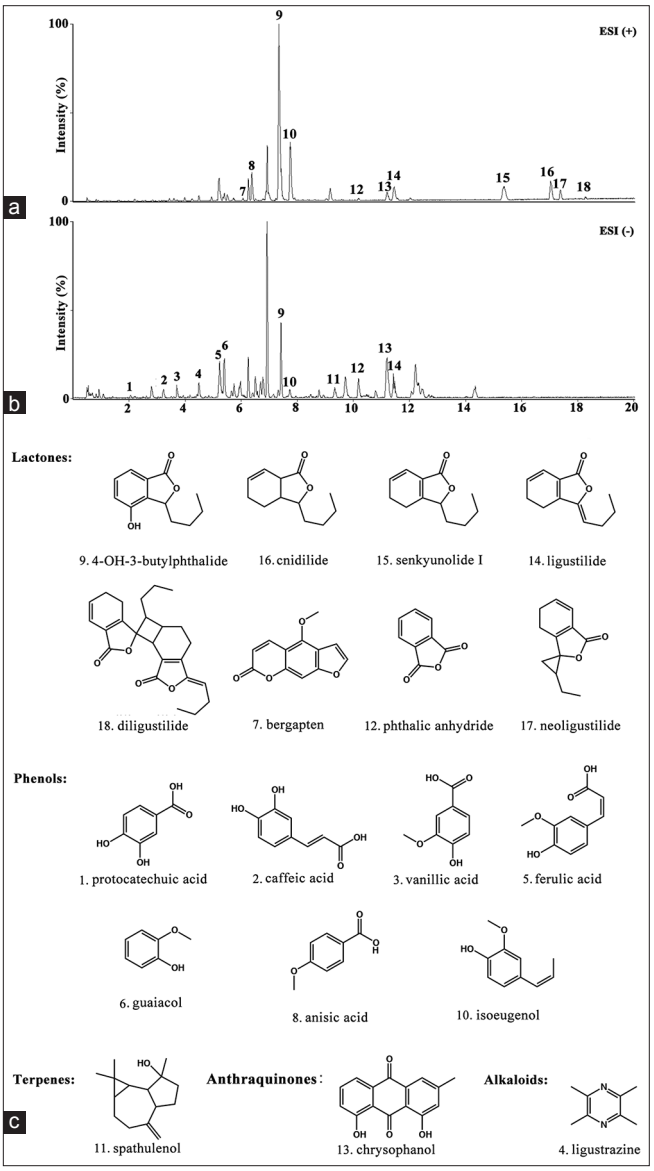

Figure 3: Ultra-performance liquid chromatography-quadrupole time-of-flight analysis of Shunaoxin pills. Base peak intensity (BPI) chromatograms in positive electrospray ionization mode (a) and in negative electrospray ionization mode (b). Structural formula of components identified (c)

Table 1: MS data in (±) ESI modes and the identification results for SNX

tR (min)	Identification	Mode	m/z	MS/MS	Composition	Source	Absorbable capacity	Targets* (Fit value)
2.06	Protocatechuic aicd	Neg	153.0182	153[M-H]-; 109 [M-H-COOH]-	$C_7H_6O_4$	Ang	√	PTGS2 (1), PTGS1 (0.425), CA1 (0.408), ADRB2 (0.355) CA2 (0.353), ADRA1A (0.342) ADRB1 (0.321)
3.22	Caffeic acid	Neg	179.0344	179[M-H]-; 135 [M-H-COOH]-	$C_9H_8O_4$	Ang	√	PTGS2 (0.759), NOS3 (0.504), PTGS1 (0.433), ADRB1 (0.351)
3.7	Vanillic acid	Neg	167.0337	167[M-H]-; 149[M-H-H2O]-; 123 [M-H-COOH]-	$C_8H_8O_4$	CX	√	PTGS2 (1), CA1 (0.685), CA2 (0.642), ADRA1A (0.463) PTGS1 (0.397) ADRB2 (0.373)
4.45	Ligustrazine	Neg	135.0455	135[M-H]-	$C_8H_{12}N_2$	Ang	√	PTGS2 (0.368); ADRA2A (0.328)
5.2	Ferulic acid	Neg	193.0498	193[M-H]-; 178 [M-H-CH3]-	$C_{10}H_{10}O_4$	Both	√	PTGS2 (0.538), OPRD1 (0.421)
5.39	Guaiacol	Neg	123.0459	123[M-H]-	$C_7H_8O_2$	CX	√	PTGS2 (0.674) ADRA1A (0.472), PTGS1 (0.445)
6.08	Bergapten	Pos	217.0501	217 [M + H]+; 189[M + H-CO]+	$C_{12}H_8O_4$	CX	√	PTGS2 (0.639)
6.38	Anisic acid	Pos	153.0541	153[M + H]+; 137[M + H-CH3]+	$C_8H_8O_3$	CX	√	PTGS2 (1), PTGS1 (0.441), CA1 (0.348), CA2 (0.323)
7.35	4-OH-3-butylphthalide	Pos	207.1044	207[M + H]+; 189[M + H-H2O]+; 177[M + H-C2H5]+	$C_{12}H_{14}O_3$	Ang	√	PTGS2 (1); PTGS1 (0.377)
7.73	Isoeugenol	Neg	163.1138	163[M-H]-	$C_{10}H_{12}O_2$	CX	√	PTGS2 (0.636)
9.35	Spathulenol	Neg	219.0649	219[M-H]-	$C_{15}H_{24}O$	Ang	√	-
10.19	Phthalic anhydride	Pos	149.1343	149[M + H]+	$C_8H_4O_3$	CX	√	PTGS2 (1), PTGS1 (0.447)
11.2	Chrysophanol	Neg	253.0714	253[M-H]-; 219[M-H-2OH]-; 205[M-H-2OH-CH3]-	$C_{15}H_{10}O_4$	Ang	√	PTGS2 (1), PTGS1 (0.443)
11.45	Ligustilide	Pos	191.1089	191[M + H]+; 149[M + H-C3H6]+	$C_{12}H_{14}O_2$	Both	√	PTGS2 (1), PTGS1 (0.397)
15.37	Senkyunolide I	Pos	193.1247	193[M + H]+; 147[M + H-H2O-CO]+	$C_{12}H_{16}O_2$	Ang	√	PTGS2 (1); PTGS1 (0.418)
17.09	Cnidilide	Pos	195.1397	195[M + H]+; 149[M + H-H2O-CO]+	$C_{12}H_{18}O_2$	Both	√	ADRB2 (0.573); CA1 (0.484); ADRB1 (0.454); CA2 (0.413)
17.38	Neoligustilide	Pos	191.1089	191[M + H]+; 149[M + H-C3H6]+	$C_{12}H_{14}O_2$	CX	√	-
18.27	Diligustilide	Pos	381.2098	381[M + H]+	$C_{24}H_{28}O_4$	Ang	-	-

*Fit value: Prediction of the antioxidant target norm fit value ≥0.3

As shown in Figure 4, cnidilide, anisic acid, vanillic acid, and protocatechuic acid may be regulated nitrogen metabolism by modulating CA1 and CA2; isoeugenol, guaiacol, protocatechuic acid, vanillic acid, chrysophanol, ligustilide, anisic acid, cnidilide, bergapten, caffeic acid, phthalic anhydride, senkyunolide I, 4-OH-3-butylphthalein, ferulic acid, and ligustrazine could be related to arachidonic acid metabolism by modulating PTGS1 and PTGS2; and protocatechuic acid, guaiacol, vanillic acid, caffeic acid, anisic acid, ligustrazine, cnidilide, and ferulic acid were predicted to regulate the cGMP-PKG signaling pathway by modulating ADRA1A, ADRA2A, ADRB1, ADRB2, OPRD1, and NOS3. The studies on Rhizoma Chuanxiong and Angelica have reported that essential oils could inhibit the production of free radical by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) ABTS detections, and that alkaloids could scavenge DPPH, nitric oxide (NO), lipid peroxidation and superoxides in the treatment of Alzheimer's diseas.[22] In addition, ferulic acid and similar phenolic compounds greatly reduce free-radical damage in neuronal cell systems without causing cell death.[23]

It has been reported that oxidative stress impairs the ability of NO to increase cGMP and the ability of cGMP to activate its specific kinase PKG by reducing the synthesis of SOD.[24] Antioxidants have also been reported to take part in the regulation of guanylate cyclase and stabilization of NO as well as to increase the levels of cGMP by stimulating guanylate cyclase.[25] Arachidonic acid effectively improves SOD and CAT activities in hippocampal tissue as well as its neuroprotective effects against oxidative stress injury induced by glutamate or H_2O_2.[26] However, there are few studies on the mechanisms of nitrogen metabolism in the human body. All of these results suggest that SNX plays a role in protecting the brain against chronic cerebral ischemia injury through antioxidant pathways. However, the precise ingredients, targets, and mechanisms were not clear.

Antioxidants screen by postcolumn-derived high -performance liquid chromatography-ultraviolet -fluorescence detectors system

Antioxidants can directly react with lipid chain oxidation intermediates, lipid free radicals, or oxygen free radicals to terminate the chain reaction and remove free radicals by inhibiting lipid oxidation.[27] Antioxidants can also affect oxidases in organisms, such as NOS, to inhibit the production of free radicals. *In vivo*, antioxidant enzymes can clear free radicals, which have important physiological functions and antioxidants can enhance the activity of antioxidant enzymes.[28] In addition to the biological antioxidant processes, there is another way to eliminate H_2O_2 directly through phenolic hydroxyl as antioxidants react with free radicals.[29]

To evaluate the chemical antioxidant activity of SNX components *in vitro*, the postcolumn-derived HPLC-UV-FLD system was established to detect the reactive oxygen radical scavenging ability based on PHPAA chemiluminescence. PHPAA can react with H_2O_2 and be converted into 2,2'-dihydroxydiphenyl-5,5'-iminodiacetic acid, which has fluorescent characteristics.[30] As shown in Figure 5, we found that two strong antioxidants from SNX, ligustilide and ferulic acid, reacted with H_2O_2 to remove oxygen free radicals significantly. It has been reported that ferulic acid and ligustilide can pass through the blood–brain barrier into the cerebrospinal fluid.[31,32] The result indicated that ligustilide and ferulic acid may be the core antioxidants in SNX.

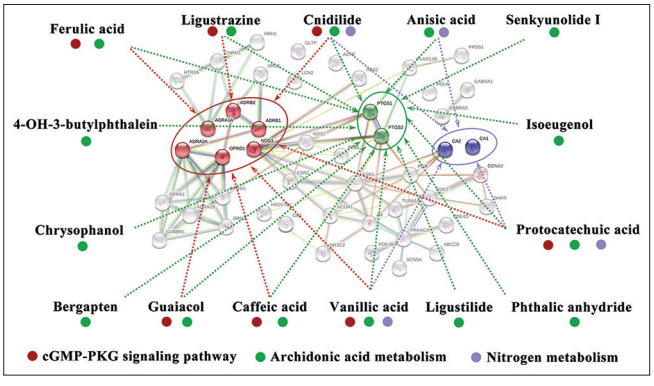

Figure 4: Network pharmacology analysis of Shunaoxin pills for antagonizing oxidative damage

Verification of the antioxidant effects of the key ingredients

The common markers of oxidative stress in the body include NO and MDA. NOS, which can produce superoxides, was shown to be expressed in neurons and postsynaptic sites of excitatory synapses.[33] Previous results demonstrated that the active ingredients could produce an antioxidant effect by reducing the content of MDA, increasing the activities of NO, NOS, and SOD. Hence, we analyzed the benefit of main antioxidants, ferulic acid and ligustilide in the biological processes. As shown in Figure 6, ferulic acid and ligustilide significantly reduced the content of MDA as well as increased the activities of NO, NOS, and SOD in a dose-dependent manner, consistent with the predicted results. As a result, ferulic acid and ligustilide not only as a chemical antioxidant, but also have the potential of biological antioxidant activity through arachidonic acid metabolism and the cGMP-PKG signaling pathway by targeting PTGS1, PTGS2, and OPRD1 multitargets. Hence, ferulic acid and ligustilide can be recommended as the Q-marker for quality control.

The intrinsic multicomponent and multitarget feature of TCM is different from the Western medicine. However, TCM is investigated essentially as herbal medicine or natural product, and the quality control are actually chemical markers based, it has deviated from the basic TCM theory.[34] In this study, chemical and biological methods were integrated to rapidly discover key components related to TCM efficacy. This approach provides a new means for the discovery of Q-markers.

Conclusion

In this study, we explored the beneficial effects of SNX for protecting cerebral tissue and speculated that the protective

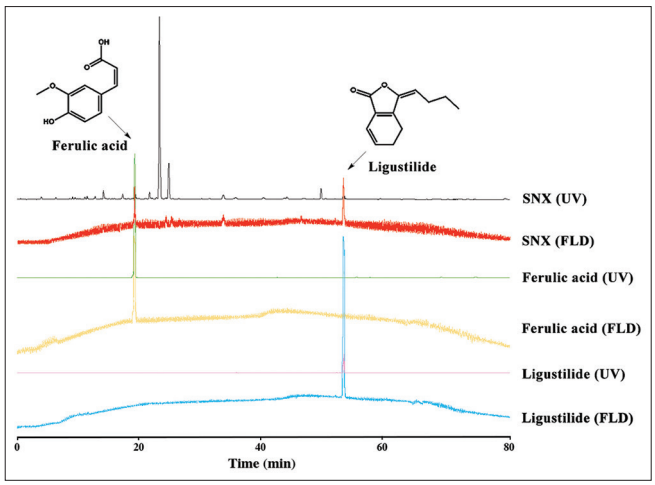

Figure 5: High-performance liquid chromatography-ultraviolet-fluorescence detectors chromatograms of Shunaoxin pills and two chemical antioxidants found in Shunaoxin pills that can scavenge H_2O_2

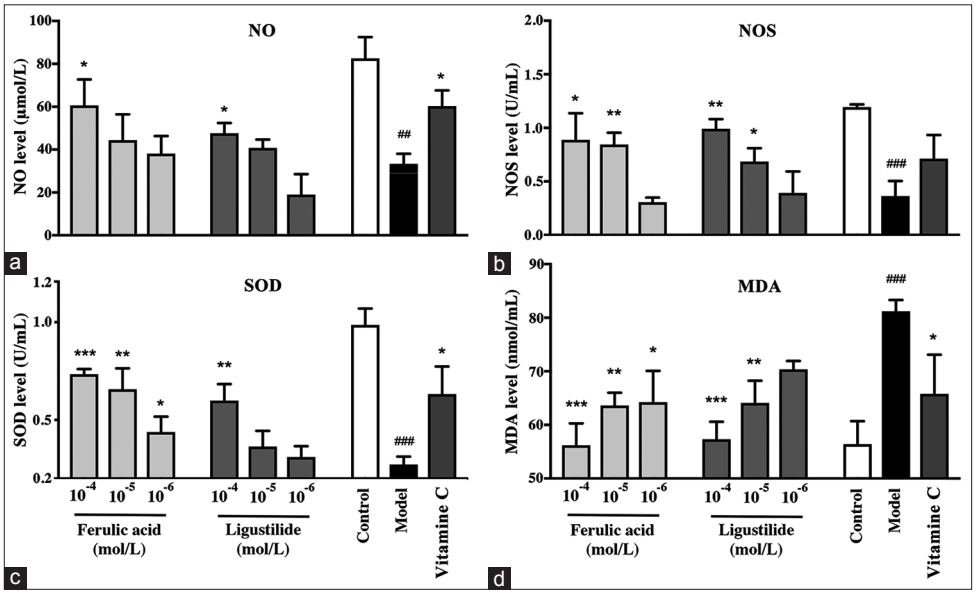

Figure 6: Confirmation of the active monomers in oxidative stress indexes. The effect of ferulic acid and ligustilide on regulating NO (a), NOS (b), SOD (c) and MDA (d) expression. Each bar represents the mean ± SD ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the model group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ the model group compared to the control group

effects in the brain were related to three main antioxidant pathways regulated by 15 components through 10 potential targets by network pharmacology. Integrated with a chemical antioxidant evolution system and cell-level oxidative stress test, we proposed that ferulic acid and ligustilide are major active ingredients as Q-markers that can be used to control the quality of SNX for treatment chronic cerebral ischemia.

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Conflicts of interest

There are no conflicts of interest.

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