RESEARCH ARTICLE

Kai Xin San ameliorates scopolamine-induced cognitive dysfunction

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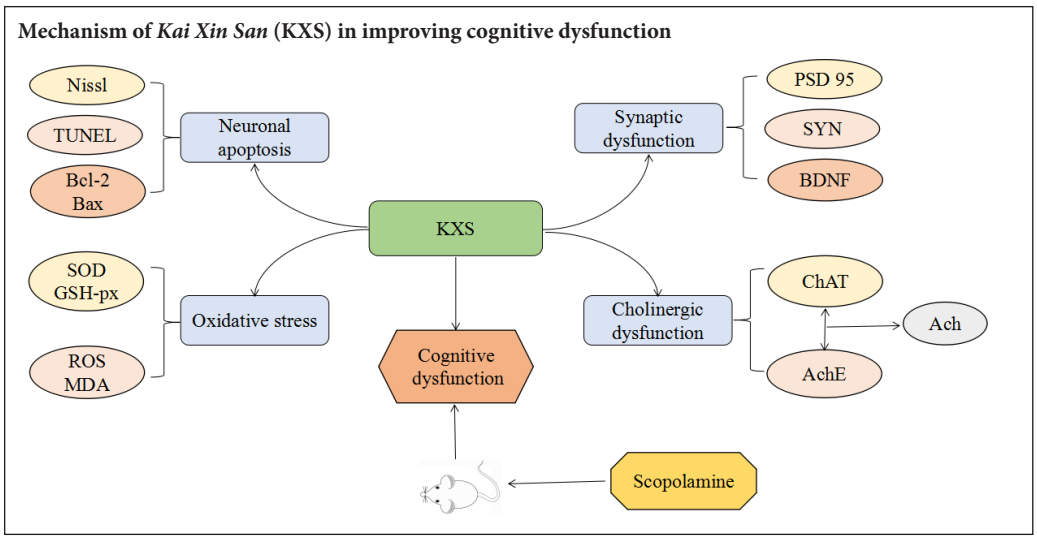
Funding: *This study was supported by the National Natural Science Foundation of China, No. 81473740, 81673627, 81673717 (to QW); Guangzhou Science Technology and Innovation Commission Technology Research Projects, China, No. 2018050100 (to QW); the Foundation for Characteristic Innovation of Educational Commission of Guangdong Province, China, Grant No. 2016KTSCX011 (to SHF); the Open Tending Project for Construction of High-Level University, Guangzhou University of Chinese Medicine, China, No. 34 and 118, 2017 (to SHF); the Technology Platform of Clinical Trials on New Traditional Medicine, China, No. 2012ZX09303009-003 (to WXL); the Technology Platform of Clinical Evaluation on New Traditional Medicine, China, No. 2008ZX09312-021 (to WXL).*

Graphical Abstract



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doi: *10.4103/1673-5374.249227*

Received: *May 14, 2018*
Accepted: *September 7, 2018*

Abstract

Kai Xin San (KXS, containing ginseng, hoelen, polygala, and acorus), a traditional Chinese herbal compound, has been found to regulate cognitive dysfunction; however, its mechanism of action is still unclear. In this study, 72 specific-pathogen-free male Kunming mice aged 8 weeks were randomly divided into a vehicle control group, scopolamine group, low-dose KXS group, moderate-dose KXS group, high-dose KXS group, and positive control group. Except for the vehicle control group and scopolamine groups (which received physiological saline), the doses of KXS (0.7, 1.4 and 2.8 g/kg per day) and donepezil (3 mg/kg per day) were gastrointestinally administered once daily for 2 weeks. On day 8 after intragastric treatment, the behavioral tests were carried out. Scopolamine group and intervention groups received scopolamine 3 mg/kg per day through intraperitoneal injection. The effects of KXS on spatial learning and memory, pathological changes of brain tissue, expression of apoptosis factors, oxidative stress injury factors, synapse-associated protein, and cholinergic neurotransmitter were measured. The results confirmed the following. (1) KXS shortened the escape latency and increased residence time in the target quadrant and the number of platform crossings in the Morris water maze. (2) KXS increased the percentage of alternations between the labyrinth arms in the mice of KXS groups in the Y-maze. (3) Nissl and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining revealed that KXS promoted the production of Nissl bodies and inhibited the formation of apoptotic bodies. (4) Western blot assay showed that KXS up-regulated the expression of anti-apoptotic protein Bcl-2 and inhibited the expression of pro-apoptotic protein Bax. KXS up-regulated the expression of postsynaptic density 95, synaptophysin, and brain-derived neurotrophic factor in the cerebral cortex and hippocampus. (5) KXS increased the level and activity of choline acetyltransferase, acetylcholine, superoxide dismutase, and glutathione peroxidase, and reduced the level and activity of acetyl cholinesterase, reactive oxygen species, and malondialdehyde through acting on the cholinergic system and reducing oxidative stress damage. These results indicate that KXS plays a neuroprotective role and improves cognitive function through reducing apoptosis and oxidative stress, and regulating synapse-associated protein and cholinergic neurotransmitters.

Key Words: *Kai Xin San; cognitive dysfunction; scopolamine hydrobromide; neuroprotection; oxidative stress; synaptic dysfunction; apoptosis; cholinergic system dysfunction; donepezil; neural regeneration*

Chinese Library Classification No. *R453; R363; R741*

Introduction

Cognitive dysfunction (CD) is a degenerative disease that is characterized by abnormalities in learning, memory, speech, execution, calculation, and comprehension (Ringman et al., 2009; Malek-Ahmadi, 2016). Learning and memory impairment and abnormal mental behaviors can affect patients' daily life and social abilities (Petersen, 2004; Tschanz et al., 2006; Boyle et al., 2010; Zhang, 2016). The etiology and pathogenesis of CD are still unclear. One major hypothesis for CD is the cholinergic hypothesis, which states that dysfunction of acetylcholine (ACh) receptors and pathways underlie learning and memory problems (Mufson et al., 2008; Chen et al., 2014). The most common drugs administered to improve cognitive function are cholinesterase inhibitors, glutamate receptor antagonists, and calcium channel blockers. However, existing drugs only relieve symptoms and cannot delay or reverse the progression of cognitive dysfunction (Petersen et al., 2005; Feldman et al., 2007; Winblad et al., 2008). Neuronal degeneration involves a variety of pathophysiological processes, and the Chinese herbal compounds can improve learning and memory in scopolamine (SCOP)-induced models of CD (Zhang et al., 2017a). The unique advantages of Traditional Chinese Medicine, including multi-target effects, might confer broader application prospects and curative effects on cognitive dysfunction (Lin et al., 2015; Dong et al., 2016a).

Kai Xin San (KXS) comprises ginseng (Panax ginseng C.A. Meyer), hoelen (Wolfiporia cocos), polygala (Polygala tenaifolia Willd), and acorus (Acorus tatarinowii Schott). KXS can benefit heart *Qi* and eliminate phlegm for resuscitation. Furthermore, KXS has been found to ameliorate cognitive dysfunction and reduce neuronal damage in human experiments and animal studies (Dong et al., 2016b; Qiong et al., 2016; Lu et al., 2017). Simultaneously, KXS has been shown to have the positive effects on improving spatial learning and memory (Hu et al., 2013b). According to the medication rules for amnesia and dementia, ginseng, polygala, hoelen, and acorus located in the positions of 1, 2, 4, and 10, respectively, in a list of high-frequency medicines used in traditional Chinese formulas, and KXS can be considered as the basic prescription for cognitive dysfunction. Ginseng, polygala, hoelen, and acorus are frequently used to treat amnesia and dementia in traditional medicine (Ji et al., 2006). KXS has been found to increase ACh levels, reduce acetylcholinesterase (AChE) activity (Dang et al., 2009), enhance superoxide dismutase (SOD) activity, reduce malondialdehyde (MDA) concentration (Hu et al., 2013a), and promote the up-regulation of brain-derived neurotrophic factor (BDNF) (Dong et al., 2016b), postsynaptic density-95 (PSD95), and synaptophysin (SYN) (Zhu et al., 2016).

The mechanisms underlying cognitive dysfunction have been proposed to include dysfunction of basal forebrain cholinergic neurons and AChE activity, and decreased levels of choline acetyltransferase (ChAT) and ACh (Ray et al., 2015; Richter et al., 2017). The therapeutic effect of donepezil, a selective AChE inhibitor, is achieved through the inhibition of reversible AChE-induced ACh hydrolysis and increasing ACh levels (Salloway et al., 2004; Schuff et al., 2011). Previous studies have found that donepezil has a regulatory effect on intracerebral cholinergic neurotransmitters, and, subsequently, on senescence-accelerated mouse prone 8 (Zhang et al., 2017b), and in a SCOP-induced mouse model (Xu et al., 2016; Zhang et al., 2017a).

SCOP can block brain information transmission and interfere with short-term memory formation (Beatty et al., 1986; Kopelman and Corn, 1988; Xiang et al., 2012). Scopolamine with intraperitoneal injection can cause learning and memory deficits, cerebral cholinergic neurotransmitter dysfunction, oxidative stress injury, and other pathological changes. These changes, which are seen following scopolamine administration in mice, simulate cognitive dysfunction (Kulshreshtha and Piplani, 2016).

Because of the complex mechanisms underlying cognitive dysfunction, it has not been possible to develop appropriate behavioral tests or biochemical indexes. This could be linked to TCM and KXS in particular. In this study, male Kunming mice were orally administered KXS to assess the effects on SCOP-induced learning and memory impairments and to further explore the underlying mechanisms of KXS in ameliorating cognitive deficits.

Materials and Methods

Preparation of KXS

All KXS herbs (acorus, ginseng, hoelen, and polygala) were acquired from the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China). The ratio of ginseng, hoelen, polygala, and acorus was 3:3:2:2. The extraction procedure shown in **Figure 1** is in accordance with previous methods for details (Hu et al., 2010). Acorus was added to six parts water as a solvent for 2 hours, followed by heat reflux extraction for 8 hours. Volatile oil and acorus dregs were saved. Ginseng was extracted twice by 60% ethanol as solvent, each time for 1 hour. The extracts were combined and filtrated, and the ginseng dregs were saved. The dregs of acorus, ginseng, hoelen, and polygala were added to 10 parts water and extracted twice, for 1 hour each. All extracts were combined and evaporated on a rotary evaporator. Finally, volatile oil of acorus and original liquid after concentrated were mixed and refrigerated at −20°C.

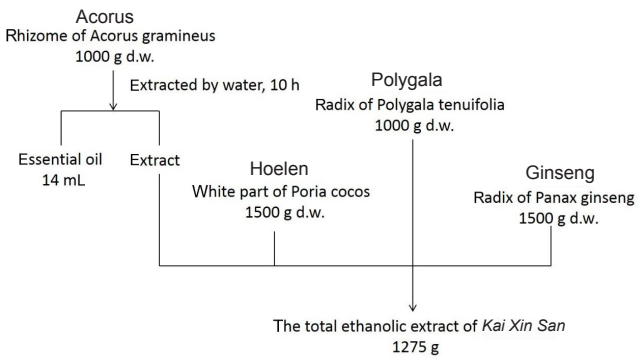

Figure 1 Extraction procedure of *Kai Xin San*.
d.w.: Delivered weight; h: hours.

The quality of KXS was controlled in accordance with a previous study (Hu et al., 2013b).

Animals

Seventy-two specific-pathogen-free male 8-week-old Kunming mice weighing 35–40 g were fed and maintained at 22 ± 2°C in a 12-hour light and dark cycle. This study was approved by the Experimental Animal Ethics Committee of Guangzhou University of Chinese Medicine, China in March 2017 (approval number: 20170315001). Experimental procedures were carried out according to the Guiding Principles for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All mice were obtained from the Experimental Animal Center of Guangzhou University of Chinese Medicine, China (license No. SCXK (Yue) 2013-0034).

Drug administration

Donepezil (Eisai Pharmaceutical Co., Ltd., China) was dissolved in 0.9% sodium chloride (GYZZ No. H44020185) and orally administered. Scopolamine hydrobromide (Aladdin Company, Shanghai, China) was dissolved in 0.9% sodium chloride and given by intraperitoneal injection. All mice were randomly divided into one of the following six groups: vehicle control group (0.9% sodium chloride, n = 12), SCOP group (3 mg/kg per day, n = 12), positive control group (SCOP 3 mg/kg per day + donepezil 3 mg/kg per day, n = 12), low-dose KXS group (SCOP 3 mg/kg per day + KXS 0.7 g/kg per day, n = 12), moderate-dose KXS group (SCOP 3 mg/kg per day + KXS 1.4 g/kg per day, n = 12), and high-dose KXS group (SCOP 3 mg/kg per day + KXS 2.8 g/kg per day, n = 12) (Cao et al., 2012; Zhou et al., 2012). 0.9% sodium chloride, donepezil, or KXS were orally administered to mice according to the groups, once a day for 2 weeks. The experimental procedure is shown in **Figure 2**. Mice received drug administration and then completed behavioral tests (Morris water maze and Y-maze test). Behavioral tests lasted 1 week and drug administration was performed synchronously during the tests. The experiment lasted a total of 2 weeks. The doses of KXS and donepezil were selected according to previous studies (Zhou et al., 2012; Zhang et al., 2017). Mice were intraperitoneally anesthetized with 1% pentobarbital sodium (0.1 mL/10 g, calculated according to body weight; Sigma, St. Louis, MO, USA).

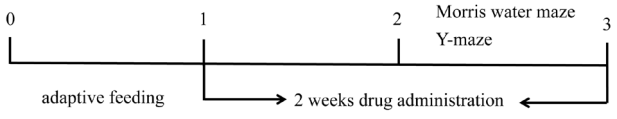

Figure 2 Experimental procedure: drug administration and behavioral tests.

Mice received drug administration after the 1-week adaptive feeding period. Behavioral tests (the Morris water maze and Y-maze spontaneous alternation test) were implemented after the 1-week drug administration period. Behavioral tests lasted 1 week, and during which drug treatment was continued. The total duration of the experiment was 2 weeks.

Morris water maze test

Morris water maze was performed after 1-week oral administration to judge the spatial learning and memory in accordance with previously reported methods (Himeno et al., 2011). The Morris water maze (Guangzhou Feidi Biology Technology Co., Ltd., Guangzhou, China) included a round pool (120 cm in diameter, 40 cm in height), an escape platform, and recording equipment. The pool was compartmentalized equally into four quadrants and the water was dyed with black-colored dye. Different-shaped markers were attached to the walls of the four-quadrant pool to facilitate orientation. The escape platform (circular, black, diameter of 10 cm) was hidden 2 cm under the water and was placed in the center of the fourth quadrants. In the Morris water maze, mice must learn to find the hidden platform and remember its position. In the subsequent 5-day experiments, mice try to find the hidden platform according to different markers of the pool wall and different water points. This experiment comprised adaptive training, directed navigation, and spatial exploration. Mice were placed in the pool and faced towards the side wall. The escape latency of swimming was recorded for each different starting point, four times a day. If the mouse successfully found the escape platform within 60 seconds, the monitoring system automatically recorded the actual time. If the mouse could not find the escape platform within 60 seconds, the researcher guided it to the platform and it remained there for 10 seconds. The platform was withdrawn after the directed navigation trials to conduct the spatial exploration part. In short, a software system (EthoVision 2.0; Noldus Information Technology, Leesburg, VA, USA) was used to automatically track the trajectory of mice in the labyrinthine pool and to record escape latency, swimming trajectories, time spent in the target quadrant, crossing times of the platform, and swimming speed.

Y-maze spontaneous alternation test

The Y-maze spontaneous alternation test was conducted after the Morris water maze test. The Y-maze (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China) spontaneous alternation test was performed as reported by Kim et al. (2011), and measures working memory. The labyrinth consists of three arms and a connecting area; the three arms have equal angles of 120°, arm lengths of 35 cm, arm widths of 5 cm, and arm heights of 15 cm. The test consisted of two phases with 1-hour intervals. During the training, the new arm was separated with a baffle and the mice were placed into the starting arm for 10 minutes, during which they had free access to the starting arm and the other arm. The test was performed 1 hour after the training. For this, the baffle in the new arm was removed and the mice were placed into the starting arm. The number of mice access into the three arms within 10 minutes was recorded. An alternation was recorded when mice made consecutive visits to the three different arms. The Y-maze spontaneous alternation was calculated as follows: Alternation behavior (%) = number of alternations/(total arm entries − 2) × 100%. At the end of the experiment, arms were cleaned with alcohol so as to remove the scent of previous mice.

Nissl staining

The mice were anesthetized and decapitated (n = 4) immediately after behavioral experiments. The brain slices were placed in an oven at 60°C for 2 hours, dewaxed with xylene, and dehydrated through a graded alcohol series. The slices were immersed in Nissl's staining solution (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China) and the staining time (5–10 minutes) was adjusted. Afterwards, slices were washed clean in running water and double-distilled water, dehydrated through a graded alcohol series (70%, 95%, and 100% alcohol) and made transparent by xylene. The distribution of neurons and staining of Nissl bodies were observed. Images were captured and analyzed at 200× magnification with a light microscope (Leica QWin Plus, Leica Microsystems, Wetzlar, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining

The mice (n = 4) were anesthetized and decapitated immediately after behavioral experiments. TUNEL staining was conducted with the In situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). The slices were placed in an oven at 60°C for 2 hours. Xylene was used for dewaxing, and graded alcohol was applied until slices were hydrated. Afterwards, the brain paraffin slices were cleaned with phosphate buffered saline and dipped in the TUNEL reaction mixture. The converter-POD was applied to the surface of slices in the wet boxes and kept away from light. The slices were treated with 3,3′-diaminobenzidine substrate and stained with hematoxylin. Images were captured and analyzed at 200× magnification with a light microscope (LEICA QWin Plus; Leica Microsystems).

Western blot assay

The mice (n = 8) were anesthetized and decapitated immediately after behavioral experiments. The cortex and hippocampus of each group were weighed and homogenized with lysis buffer as well as adding to 1 mM phenylmethyl sulfonylfluoride and a protease inhibitor cocktail. The homogenate was centrifuged at 3000 × g for 10 minutes at 4°C. According to the protein standard of 30 μg per well, the protein concentration was calculated, and the sodium dodecyl sulphate lysate was mixed. The loading buffer was applied to adjust the uniform concentration. The sample protein was denatured by boiling for 10 minutes at 100°C. The sample protein of each group was detached by sodium dodecyl sulfate polyacrylamide gel electrophoresis and converted to the polyvinylidene difluoride membranes. The membranes were soaked in 5% bovine serum albumin for 1.5 hours at room temperature. The membranes were then soaked overnight at 4°C with one of the following primary anti-bodies: rabbit anti-antiapoptotic Bcl-2, rabbit anti-proapoptotic Bax, rabbit anti-PSD95 (1:1000; PSD95 participates in the regulation of the number of synapses and promotes the formation of synapses), rabbit anti-SYN (1:1000; SYN reflected with the synaptic density), rabbit anti-BDNF (1:1000; BDNF promotes the differentiation, growth and survival of neurons) and mouse anti-β-actin. Subsequently, the polyvinylidene difluoride membranes were soaked in horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:4000) at room temperature for 2 hours. After rinsing on the shaker with Tris-buffered saline with Tween, the membranes were placed on the surface of the gel imager by applying the super-enhanced chemiluminescence western-blot analysis-detection reagent (Applygen Technologies Inc., Beijing, China). β-Actin was used as an internal control. Intensity of the bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The results are presented as the ratio between the intensity of target proteins. All the antibodies were purchased from Abcam, Cambridge, MA, USA.

Biochemical analysis

The mice (n = 8) were anesthetized and decapitated immediately after behavioral experiments. The activities of ChAT, AChE, and ACh were detected using assay kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China). The cortex and hippocampus of each group were pretreated with 0.9% sodium chloride. The homogenate was centrifuged at 3000 × g at 4°C for 10 minutes. The supernatant liquid was stored in Eppendorf tube, and the levels and activities of ACh, AChE, and ChAT were detected according to the instruction manual with a Universal Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA).

The mice (n = 8) were anesthetized and decapitated immediately after behavioral experiments. The activities of SOD, glutathione peroxidase (GSH-Px), and MDA were detected using assay kits (Nanjing Jiancheng Institute of Biotechnology). The cortex and hippocampus of each group were pretreated with 0.9% sodium chloride. The homogenate was centrifuged at 3000 × g at 4°C for 10 minutes. The supernatant was stored in an Eppendorf tube. The activities of SOD, GSH-Px, and MDA were detected in accordance with the instruction manual applying a Universal Microplate Spectrophotometer (Bio-Rad).

The mice were anesthetized and decapitated immediately after behavioral experiments. Reactive oxygen species (ROS) contents were detected using assay kits (Nanjing Jiancheng Institute of Biotechnology). The cortex and hippocampus of each group were pretreated with 0.9% sodium chloride. The homogenate was centrifuged at 3000 × g at 4°C for 10 minutes. The organizational supernatant was stored in an Eppendorf tube, and ROS contents were detected with redox-sensitive fluorescent dye, DCFH-DA. Transformation of nonfluorescent DCFH-DA into fluorescent dichlorofluorescein in the presence of ROS was detected with a microplate reader (Bio-Rad). Fluorescence emission intensity of dichlorofluorescein at 538 nm was detected in response to 485 nm excitation. Intracellular ROS content was expressed as a percentage that of control cultures incubated in DCFH-DA.

Statistical analysis

Data, shown as the mean ± SEM, were analyzed using SPSS 19.0 statistical software (IBM, Armonk, NY, USA). One-way

analysis of variance followed by Dunnett's significant *post hoc* test was conducted for pair-wise multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

KXS ameliorates spatial learning and memory impairments

In the navigation test, the escape latency of mice decreased with an increasing number of training sessions (**Figure 3A**). However, the escape latency was shorter in the KXS groups and the positive control group compared with the SCOP group. The swimming trajectories of each group on days 1 and 5 are shown in **Figure 3B**. Mice in the SCOP group exhibited a disorderly trajectory and spent longer finding the platform, both of which were improvement after the administration of KXS and donepezil. The SCOP group spent less time in the target quadrant ($P < 0.01$; **Figure 3C**) and crossed the platform fewer times ($P < 0.01$; **Figure 3D**) than the KXS and donepezil groups. Swimming speed was slower in the SCOP group compared with the vehicle control group ($P < 0.05$; **Figure 3E**).

In the Y-maze spontaneous alternation test, the vehicle control group exhibited more spontaneous alternation compared with the SCOP group ($P < 0.01$; **Figure 3F**). The spontaneous alternations were more frequent after treatment with KXS and donepezil compared with the SCOP group ($P < 0.05$).

KXS reduces neuronal apoptosis

The hippocampal area of the SCOP group exhibited degenerated or shrunken neuronal vacuoles, few or missing Nissl's bodies, and weakly stained and low neuronal numbers (**Figure 4A**).The differences between the SCOP group and the treatment groups (KXS and positive control) were not yet clear. In the TUNEL staining (**Figure 4B**), compared with the vehicle control group, the SCOP group exhibited fewer cerebral cortex neurons and more brown apoptotic bodies. The KXS and donepezil groups showed less neuronal apoptosis than the SCOP group. In addition, the SCOP group showed a down-regulated expression of apoptosis-related protein Bcl-2 and an upregulation of Bax compared with the treatment groups (KXS and positive control) (**Figure 4C–D**). KXS and donepezil groups showed greater Bcl-2 expression and lower Bax expression than the SCOP group.

KXS reduces neuronal degeneration

Synaptic changes in the central nervous system underlie learning memory formation. As shown in **Figure 5**, the expression of PSD95, SYN and BDNF were significantly

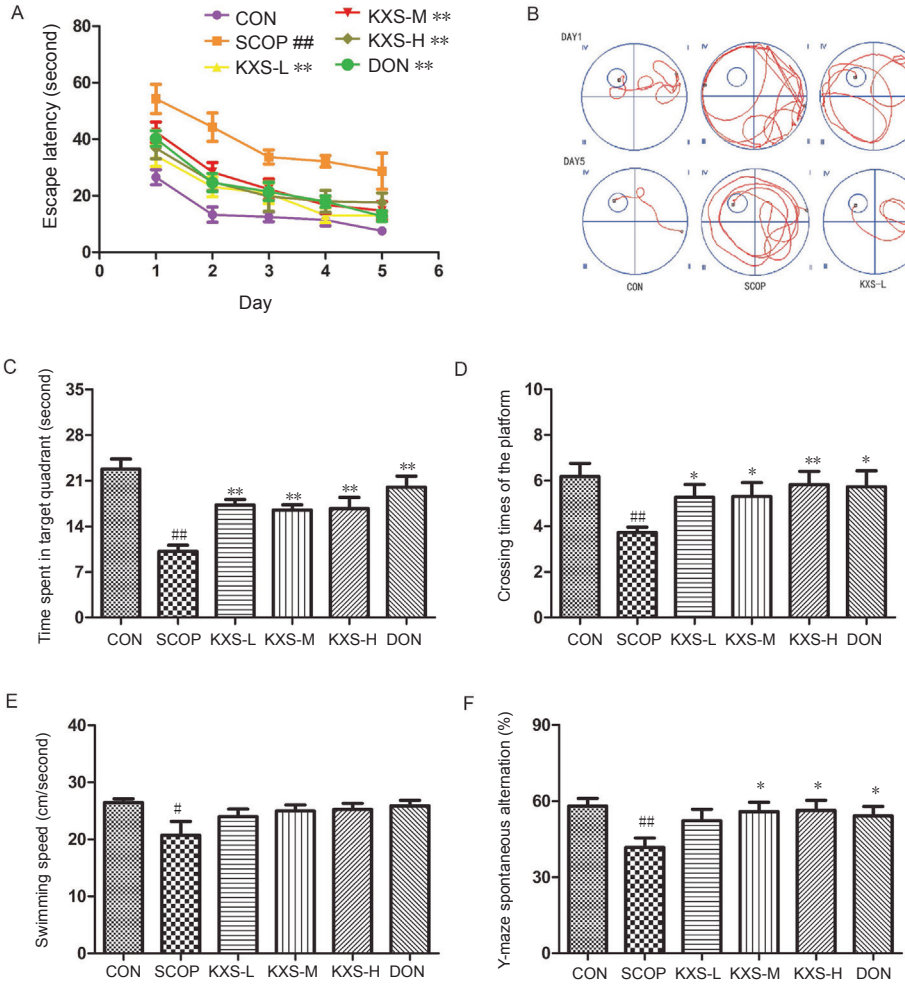

Figure 3 KXS ameliorates spatial learning and memory impairments.
Escape latency (A), swimming trajectories (B), time spent in target quadrant (C), crossing times of the platform (D), and swimming speed (E) measured in the Morris water maze test. (F) Y-maze spontaneous alternation. Data are shown as the mean ± SEM ($n = 12$; one-way analysis of variance followed by Dunnett's *post hoc* test). The experiment was performed in triplicate. #$P < 0.05$, ##$P < 0.01$, *vs.* CON group; *$P < 0.05$, **$P < 0.01$, *vs.* SCOP group. KXS: *Kai Xin San*; CON: vehicle control; SCOP: scopolamine; KXS-L: scopolamine + *Kai Xin San* (0.7 g/kg per day); KXS-M: scopolamine + *Kai Xin San* (1.4 g/kg per day); KXS-H: scopolamine + *Kai Xin San* (2.8 g/kg per day); DON: scopolamine + donepezil (3 mg/kg per day); d: days.

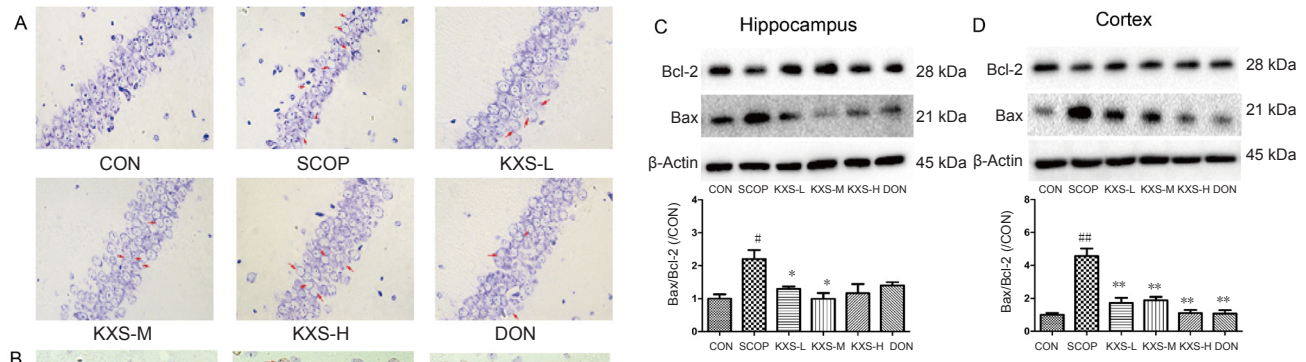

Figure 4 KXS reduces neuronal apoptosis.
(A) Nissl staining in the hippocampus: neurons were disordered and Nissl bodies in the cells were reduced or even vacuolate (red arrow; × 200). (B) TUNEL staining in the cortex and apoptotic bodes (red arrow; × 200). (C, D) Protein expression of Bcl-2 and Bax was detected in the hippocampus and cortex, respectively. Data are shown as the mean ± SEM (n = 8; one-way analysis of variance followed by Dunnett's *post hoc* test). The experiment was performed in triplicate. #P < 0.05, ##P < 0.01, *vs.* CON group; ∗P < 0.05, ∗∗P < 0.01, *vs.* SCOP group. KXS: *Kai Xin San*; CON: vehicle control; SCOP: scopolamine; KXS-L: scopolamine + Kai Xin San (0.7 g/kg per day); KXS-M: scopolamine + *Kai Xin San* (1.4 g/kg per day); KXS-H: scopolamine + *Kai Xin San* (2.8 g/kg per day); DON: scopolamine + donepezil (3 mg/kg per day). d: Day.

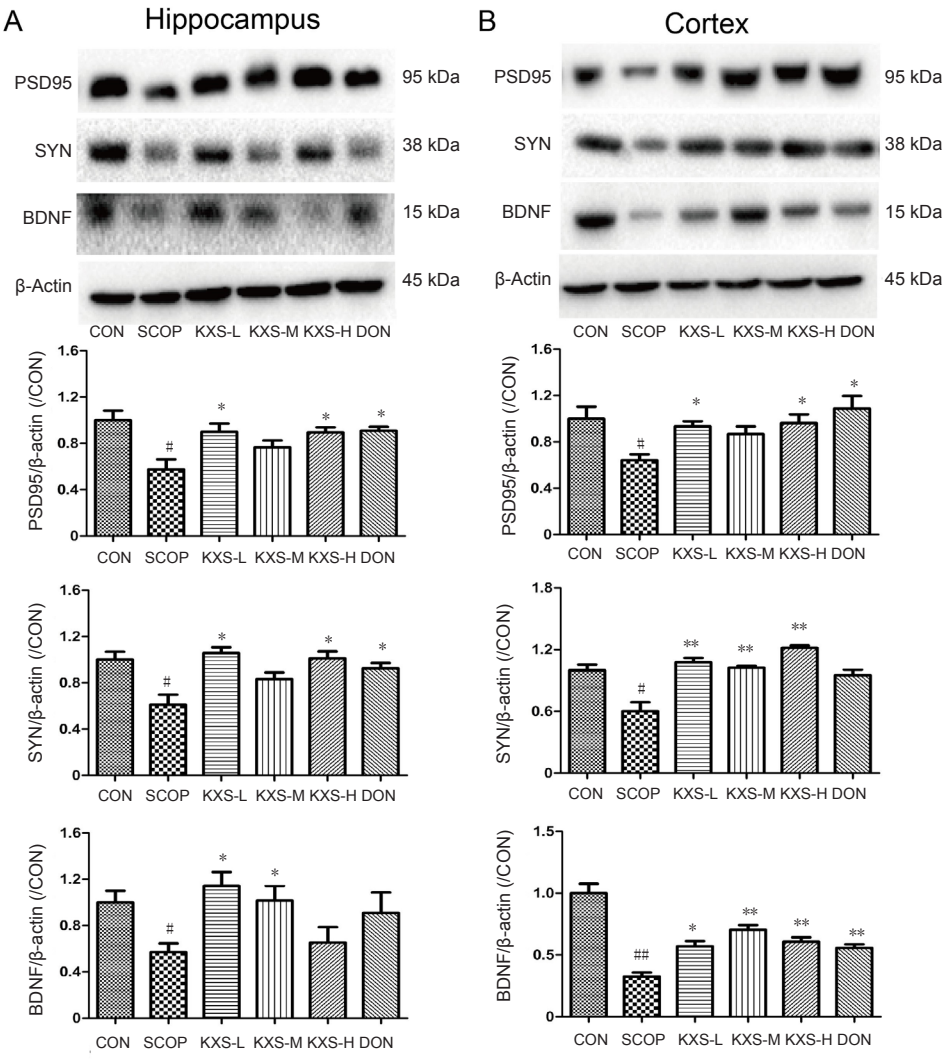

Figure 5 KXS reverses neuronal degeneration.
(A, B) Western blot assay results showed that KXS administration increased the expression of PSD95, SYN, BDNF in the hippocampus and cortex compared with the model (SCOP) group, respectively. Data are shown as the mean ± SEM (n = 8; one-way analysis of variance followed by Dunnett's *post hoc* test). The experiment was performed in triplicate. #P < 0.05, ##P < 0.01, *vs.* CON group; ∗P < 0.05, ∗∗P < 0.01, *vs.* SCOP group. KXS: *Kai Xin San*; CON: vehicle control; SCOP: scopolamine; KXS-L: scopolamine + *Kai Xin San* (0.7 g/kg per day); KXS-M: scopolamine + *Kai Xin San* (1.4 g/kg per day); KXS-H: scopolamine + *Kai Xin San* (2.8 g/kg per day); DON: scopolamine + donepezil (3 mg/kg per day). PSD95: Postsynaptic density-95; SYN: synaptophysin; BDNF: brain-derived neurotrophic factor; d: days.

down-regulated in the SCOP group. The KXS and donepezil groups showed a higher expression of these neurotrophic factors compared with the SCOP group ($P < 0.05$ or $P < 0.01$).

Effect of KXS on cholinergic neurotransmitters

To investigate the mechanism of action of KXS on ameliorating cognitive dysfunction, the activities of AChE and ChAT and the contents of ACh were tested (**Figure 6**). As shown in **Figure 6A–D**, the SCOP group showed significantly higher AChE activity and lower ChAT activity compared with the vehicle control group ($P < 0.05$ or $P < 0.01$). KXS and donepezil increased ChAT activity, and ACh content (**Figure 6E** and **F**), decreased AChE activity to regulate this abnormal situation.

KXS alleviates oxidative stress injury

Significantly lower activity of SOD and GSH-Px (**Figure 7A–D**) and higher ROS and MDA contents were found in the in the SCOP group ($P < 0.05$ or $P < 0.01$; **Figure 7E–H**).

Discussion

The gradual decline of learning and memory is a typical symptom of CD (Mao et al., 2017). In this study, we found that KXS reduced learning and memory impairments and anti-neuronal apoptosis, regulated the expression of synapse-associated protein and cholinergic neurotransmitters, and inhibited oxidative stress damage in the cortex and hippocampus.

Previous *in vivo* studies have reported that KXS promotes long-term potentiation, enhances synaptic plasticity, increases PSD95 expression in the hippocampus and cortex (Di et al., 2017), regulates the balance of monoamine neurotransmitters (Shi et al., 2017), and improves cognitive impairment and psychiatric symptoms in animal models of dementia (Lu et al., 2017). Previous *in vitro* studies have reported that KXS increases the synthesis and release of neurotrophic factors (nerve growth factor and BDNF) (Zhu et al., 2013), and mitigates astrocyte damage caused by corticosterone (Wen et al., 2014). In addition, the main active ingredients of the KXS herbs reportedly have a beneficial effect on cognitive function. Namely, ginsenoside Rg1 has been found to reduce Aβ deposition in the hippocampus and inhibit Aβ-induced apoptosis (Ye et al., 2017). Pachymaran alleviates spatial direction discrimination through reducing mouse brain monoamine oxidase activity (Zhang et al., 2016). β-Asarone, the main active substance of acorus,

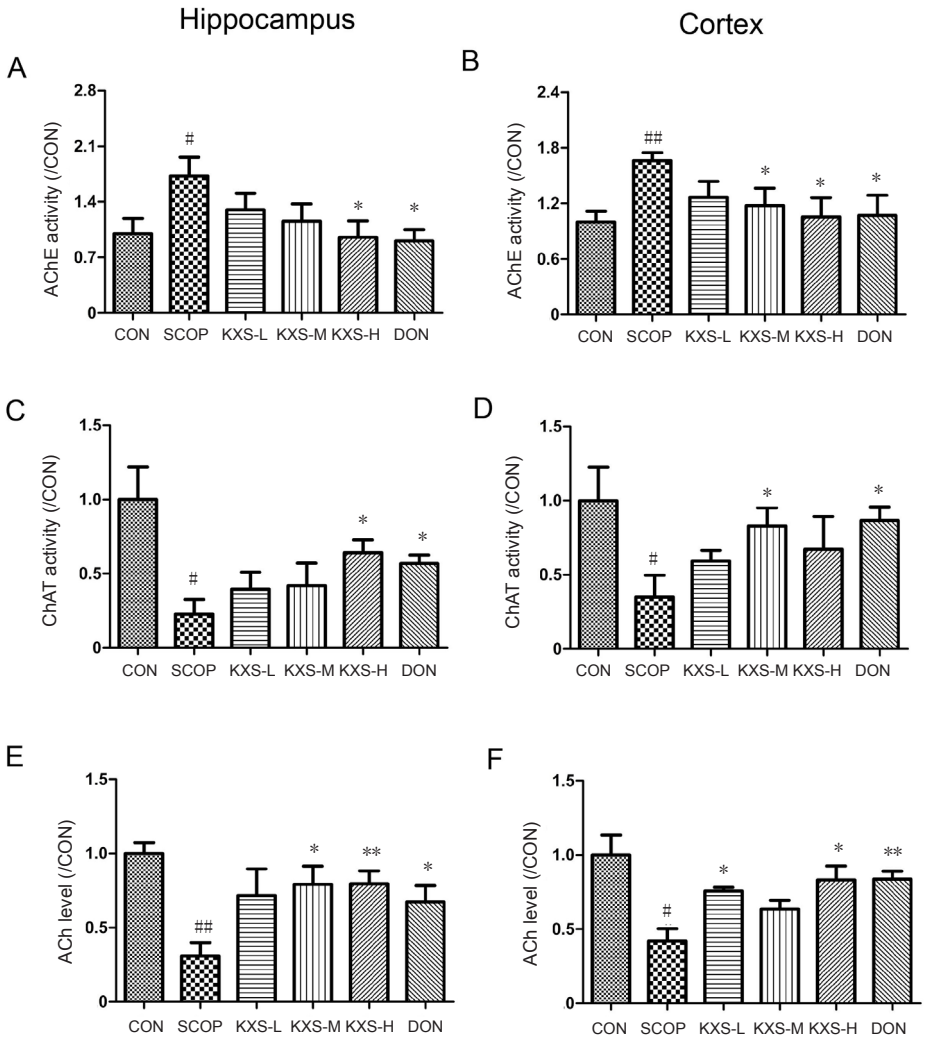

Figure 6 Effect of KXS on cholinergic neurotransmitters.

Assay detection showed that KXS administration increased the expression of ChAT and ACh, decreased the expression of AChE in the hippocampus and cortex compared with the SCOP group. AChE activities (A, B), ChAT activities (C, D), and ACh levels (E, F). Data are shown as the mean ± SEM ($n = 8$; one-way analysis of variance followed by Dunnett's *post hoc* test). The experiment was performed in triplicate. #$P < 0.05$, ##$P < 0.01$, *vs.* CON group. *$P < 0.05$, **$P < 0.01$, *vs.* SCOP group. KXS: *Kai Xin San*; CON: vehicle control; SCOP: scopolamine; KXS-L: scopolamine + *Kai Xin San* (0.7 g/kg per day); KXS-M: scopolamine + *Kai Xin San* (1.4 g/kg per day); KXS-H: scopolamine + *Kai Xin San* (2.8 g/kg per day); DON: scopolamine + donepezil (3 mg/kg per day). ChAT: Choline acetyltransferase; ACh: acetylcholine; d: days.

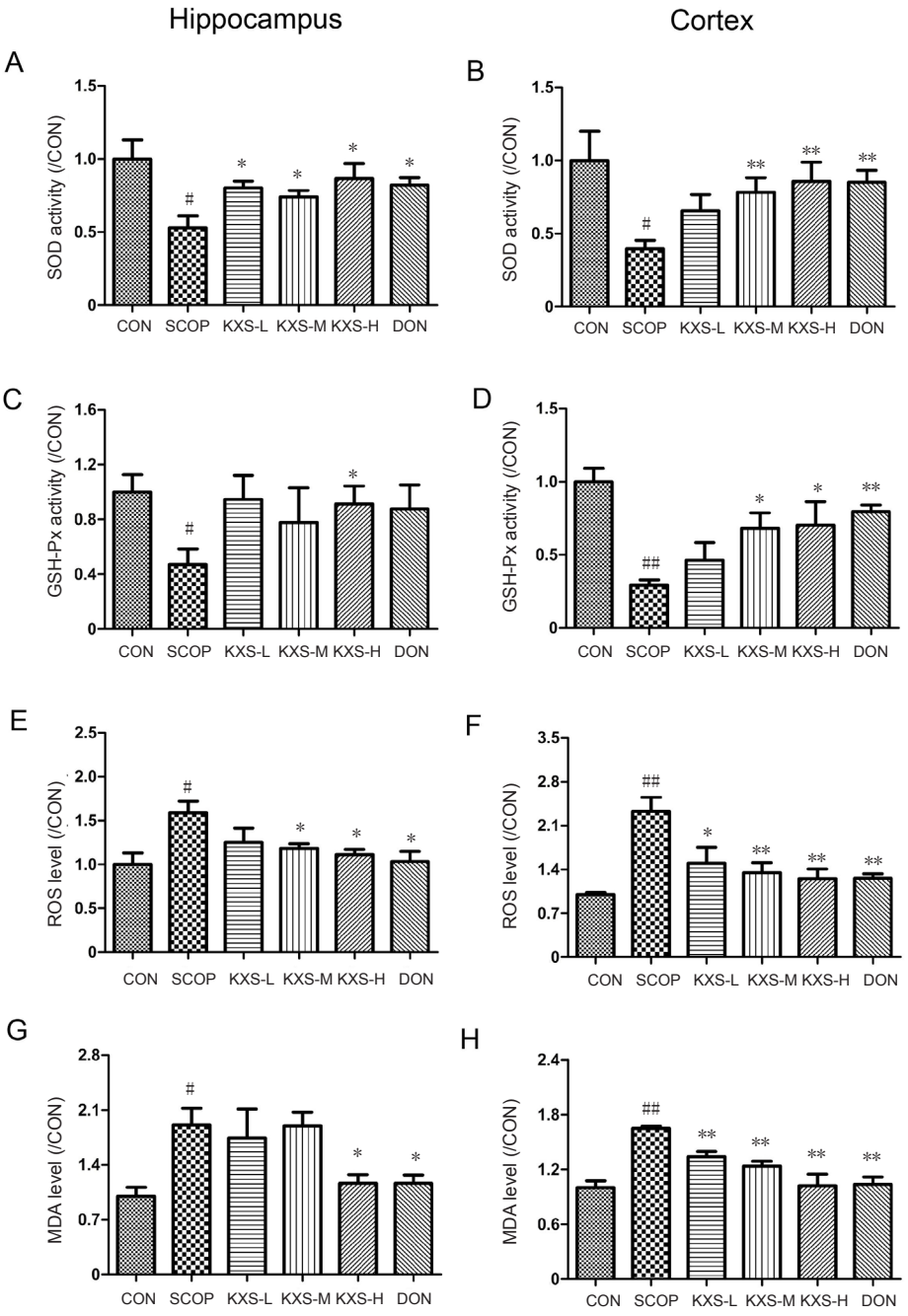

Figure 7 KXS decreases oxidative stress injury.

Assay detection showed that KXS administration increased the expression of SOD and GSH-Px, decreased the expression of ROS and MDA in the hippocampus and cortex compared with the SCOP group. SOD (A, B), GSH-Px (C, D), ROS (E, F), and MDA (G, H). Data are shown as the mean ± SEM (n = 8; one-way analysis of variance followed by Dunnett's *post hoc* test). The experiment was performed in triplicate. #P < 0.05, ##P < 0.01, *vs.* CON group. *P < 0.05, **P < 0.01, *vs.* SCOP group. KXS: *Kai Xin San*; CON: vehicle control; SCOP: scopolamine; KXS-L: scopolamine + *Kai Xin San* (0.7 g/kg/per day); KXS-M: scopolamine + *Kai Xin San* (1.4 g/kg per day); KXS-H: scopolamine +*Kai Xin San* (2.8 g/kg per day); DON: scopolamine + donepezil (3 mg/kg per day). SOD: Superoxide dismutase; GSH-Px: glutathione peroxidase; ROS: reactive oxygen species; MDA: malondialdehyde; d: day.

exerts anti-inflammatory effects by inhibiting the release of pro-inflammatory mediators through the nuclear factor-kB signaling pathway (Lim et al., 2014). Polygala extract has been found to increase the expression of insulin-degrading enzyme, enkephalinase, and BDNF by regulating the BDNF/TrkB signaling pathway to reduce Aβ deposition (Peng et al., 2017).

The Morris water maze measures spatial learning and memory functions in animal models and is widely used in neuroscience research (Morris, 1984; Brandeis et al., 1989; Kim et al., 2007). The Y-maze test measures working memory, learning, exploration ability, and spatial recognition in unfamiliar environments Previous studies have reported that KXS improves cognitive abilities in different animal models of cognitive deficits (Qiong et al., 2016; Lu et al., 2017). In this study, the mice in SCOP group presented with serious cognitive impairment.

Oxidative stress has been found to lead to lipid peroxidation, oxidation of proteins and DNA, and changes in the redox state, thereby promoting neuronal apoptosis (Jahanshahi et al., 2013; Aliev et al., 2014). Bcl-2 and Bax genes reportedly have competitive expression trends during the growth and development of neurons (Goldsmith et al., 2012). A series of apoptotic cascades activate the caspase family and eventually cause neuronal apoptosis. In this study, KXS and donepezil inhibited Bax up-regulation and promoted Bcl-2 expression,

which strengthened apoptosis modulation.

Some studies have reported that neuronal-synaptic loss is the direct cause of dementia onset and cognitive decline (Bayer et al., 2005; Holmes et al., 2008; Holtzman, 2008; Wilcock et al., 2009). In this study, we found that intervention groups (KXS and positive control groups) had varying degrees of neuronal damage. KXS and donepezil treatment up-regulated the expression of PSD95, SYN, and BDNF and reduced synaptic loss and dysfunction.

Cholinergic system dysfunction in the brain is one of the key factors underlying cognitive dysfunction (Kilimann et al., 2017). In early stages of cognitive dysfunction, cholinergic system impairments can result in decreased levels of ACh and cognitive decline (Yu et al., 2008). ACh levels depend on the balance between formation and degradation, which is strongly associated with ChAT and AChE levels (Seabrook et al., 2007). In this study, the neurotransmitters and active enzymes of the cholinergic system were detected. Compared with the SCOP group, the KXS groups exhibited higher ACh levels and ChAT activity and a decrease of AChE activity in both in the cortex and hippocampus. Therefore, KXS provided a neuroprotective effect *via* regulation of the cholinergic system. Previous studies have shown that donepezil had a similar effect of increasing ACh levels in the brain and enhancing cognitive ability (Lian et al., 2017; Sui et al., 2017).

Oxidative stress injury is the one of the factors of aging and neurodegenerative diseases. Intraperitoneal injection of scopolamine has been found to decrease antioxidant enzyme activity in animal brains (Budzynska et al., 2015). Moreover, memory impairments can be caused by blocking the transmission of cholinergic signals (Ishola et al., 2013). A previous study found that the destruction of the redox balance *in vivo* caused a decrease in SOD and GSH-Px enzyme activity and an increase of ROS, MDA, and lipid peroxidation, which led to neuronal damage and memory dysfunction (Aliev et al., 2014). In this study, KXS increased the production of SOD and GSH-Px, and reduced the expression of MDA and ROS compared with the SCOP group. These results suggest that KXS could enhance memory abilities by regulating the body's antioxidant ability and reducing oxidative damage induced by scopolamine.

It is worth noting that the adverse reactions caused by radix polygalae treatment mainly manifest as nausea, vomiting, and allergic symptoms with chest tightness or skin itching (Lin, 2011). The individual chemical compounds of radix polygalae could inhibit CYP2E1 enzyme activity and cause drug interactions. Long-term use of Chinese patent medicine or Chinese herbal compounds containing radix polygalae comes with carcinogenic and teratogenic risks (Li et al., 2014). Therefore, it is important to limit the application of radix polygalae. In addition, the research scope and application dosage of KXS in the preparation phase have been discussed. The doses in the previous studies have neuroprotective effects (Cao et al., 2012; Zhou et al., 2012).

According to the equivalent dose ratio converted from body surface between human and mouse, the largest adult daily powder for clinical application was calculated to be 21.54 g (the weight of adult 70 kg as the standard). Studies on acute and sub-chronic oral toxicity have demonstrated that, in mice, the no-observed-adverse-effect level, the median lethal dose (LD50), and the absolute lethal dose (LD100) of KXS were 19.67, 32.59, and 60.04 g/kg, respectively (Mu et al., 2011). Therefore, the dosages of KXS (0.7, 1.4, and 2.8 g/kg) used in this study (Kunming mice, 8 weeks old, 35–40 g) was safe. The minimum dose has a certain therapeutic effect and the each group of KXS is effective, but the difference between the groups is not significant. We will further explore the dose range. This study is an *in vivo* experiment and it is necessary to perform the *in vitro* experiments further supplement, explain, and verify our findings.

In conclusion, this study demonstrated the neuroprotective effect of KXS and the potential underlying mechanisms on SCOP-induced cognitive dysfunction. The beneficial effect of KXS may have been achieved through reducing neuronal apoptosis, ameliorating synaptic dysfunction, regulating cholinergic neurotransmitters, and decreasing oxidative stress. Future investigations are needed to explore the mechanism of KXS, a traditional Chinese herbal formula, on cognitive dysfunction.

Acknowledgments: *The authors are grateful to the staff from the Institute of Clinical Pharmacology of Guangzhou University of Chinese Medicine, China for providing research platforms, technical and funding support.*

Author contributions: *YMX and XCW performed the experiment and wrote the manuscript. WXL, QW and SJZ participated in the design and implementation of the research scheme. TTX, HYL, SYH and NCL performed behavioral experiments. HW, WZ, SHF, YBC, LG and YQF executed data analysis. All authors read and approved the final manuscript.*

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

Financial support: *This study was supported by the National Natural Science Foundation of China, No. 81473740, 81673627, 81673717 (to QW); Guangzhou Science Technology and Innovation Commission Technology Research Projects, China, No. 2018050100 (to QW); the Foundation for Characteristic Innovation of Educational Commission of Guangdong Province, China, Grant No. 2016KTSCX011 (to SHF); the Open Tending Project for Construction of High-Level University, Guangzhou University of Chinese Medicine, China, No. 34 and 118, 2017 (to SHF); the Technology Platform of Clinical Trials on New Traditional Medicine, China, No. 2012ZX09303009-003 (to WXL); the Technology Platform of Clinical Evaluation on New Traditional Medicine, China, No. 2008ZX09312-021 (to WXL). The funding sources had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.*

Institutional review board statement: *The animal experiment was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine, China (approval No. 20170315001). The procedures of experiment were implemented in the light of the Guiding Principles for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.*

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

Plagiarism check: *Checked twice by iThenticate.*

Peer review: *Externally peer reviewed.*

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