Research Paper

Potential Effects of Icariin on Learning Performance in a Scopolamine-Induced Cognitive Impairment Mouse Model

R.W. WANG, Y.N. BAI, L.L. HAO, X.K. DI, M.Y. WAN AND Y. LI*

School of Pharmacy, North China University of Science and Technology, Tangshan 063000, China

Icariin, one of the main active components of *Epimedium brevicornum* Maxim, exerts extensive bioactivities of anti-oxidant stress, anti-aging and anti-tumor. However, there was only a few studies on icariin's mechanisms in alleviating the cognitive impairment. The present study investigated the potential effects of Icariin on the learning performance in a scopolamine-induced cognitive impairment mouse model. Sixty sun protection factor-grade male mice were divided into six groups: Control group, scopolamine group, donepezil group and low-, medium-, and high-dose icariin groups+scopolamine. The control and scopolamine groups were given 0.5 % calcium magnesium acetate-sodium, the donepezil group was given 2 mg/kg donepezil hydrochloride, and the low-, medium-, and high-dose icariin groups were given 30 mg/kg, 60 mg/kg and 120 mg/kg icariin, respectively. All mice were given 10 ml/kg volume by continuous gavage for 14 d. The cognitive impairment mouse model was established from the 10th d, the control group was intraperitoneally injected with 0.9 % normal saline, the other groups were intraperitoneally injected with 2 mg/kg scopolamine hydrobromide (10 ml/kg) for five consecutive days within 30 min of the start of the behavioral experiment. Morris water maze task, new object recognition and dark avoidance experiment were performed to assess learning performance. Pathological changes in the hippocampal cornu ammonis 1 neurons were observed through hematoxylin-eosin staining. The levels of oxidative stress parameters malondialdehyde, superoxide dismutase, glutathione, glutathione peroxidase and cholinergic system markers acetylcholinesterase, choline-acetyltransferase and acetylcholine were detected by test kits. Additionally, the levels of brain-derived neurotrophic factor, synaptophysin, postsynaptic density-95, caspase-3, B-cell leukemia-2 associated X protein and B-cell leukemia-2 were detected by Western blots. In conclusion, the results indicate that icariin may alleviate scopolamine-induced cognitive impairment by improving oxidative stress, and regulating cell apoptosis and synaptic related protein expression.

Key words: Icariin, cognitive impairment, scopolamine, learning performance, brain-derived neurotrophic factor

In recent years, with the gradual development of aging population, the incidence of cognitive decline and neurodegenerative diseases has rapidly increased, but there is currently a lack of effective prevention and treatment measures[1]. Extensive research on the impairment of cognitive function has shown that targeting multiple pathways can be an effective therapeutic strategy for alleviating cognitive impairment and regulating brain function[2]. Natural drugs exhibit unique advantages such as high efficiency and less toxicity, which have attracted the attention of many scholars[3]. Most drugs currently used to treat cognitive impairment by regulating oxidative stress, inhibiting inflammatory response, delaying cell apoptosis[4-6], and mediating the activation of Brain-Derived Neurotrophic Factor (BDNF)/Tropomyosin receptor kinase-B (TrkB)-related pathways and expression of synaptic-related proteins[7,8].

Epimedium brevicornum Maxim is a traditional Chinese medicine with multiple effects[9]. According to the Chinese Pharmacopoeia, it is a promising

***Address for correspondence**
E-mail: yangli@ncst.edu.cn

Accepted 23 July 2024
Revised 15 February 2024
Received 31 July 2023
Indian J Pharm Sci 2024;86(4):1258-1268

pharmacological agent with roles in dispelling wind and dampness, tonifying the kidney, and enhancing bone mineral density[10], among others. Icariin (ICA) is a major active component of *Epimedium brevicornum* Maxim and belongs to flavonoids (fig. 1). Pharmacological activities of ICA have been reported in studies. For example, a study reported that ICA plays a role in increasing the activity of antioxidant enzymes and preventing oxidative stress[11]. Additionally, ICA was found to play an anti-inflammatory role by inhibiting the over activation of astrocytes[12]. Moreover, ICA could combat cytotoxicity and reduce excessive precipitation of Beta (β)-amyloid peptide, thereby alleviating cognitive impairment[13]. Accordingly, it can be inferred that ICA has the potential to alleviate cognitive impairment in mice. In this study, we used Scopolamine (SCOP) to induce cognitive impairment in mice and explored the mechanism of action of ICA in alleviating learning performance of the cognitively impaired mice.

MATERIALS AND METHODS

ICA (purity≥98 %, YYH2020-5-25) was obtained from Xi'an Shengqing Biotechnology Co., Ltd., (Xi'an, China). SCOP (S817762, CAS: 114-49-8) and donepezil hydrochloride (D849374, CAS: 120011-70-3) were obtained from Shanghai Macklin Biochemical Co., Ltd., (Shanghai, China). The kits for measuring the levels of Malondialdehyde (MDA), Superoxide Dismutase (SOD), Glutathione (GSH), GSH-Peroxidase (GSH-Px), Acetylcholinesterase (AChE), Choline-Acetyltransferase (ChAT), and Acetylcholine (Ach) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Hematoxylin and eosin were procured from Wuhan Jinhong Biotech Development Co., Ltd., (Zhuhai, China). The Postsynaptic Density-95 (PSD-95), Synaptophysin (SYN), BDNF, caspase-3, B-cell leukemia-2 Associated X protein (BAX), B-cell leukemia-2 (Bcl-2), and Beta (β)-actin antibodies were obtained from Hangzhou HuaAn Biotechnology Co., Ltd., (Hangzhou, China). Secondary antibodies, Bovine Serum Albumin (BSA) and the Enhanced Chemiluminescence (ECL) reagents were the products of Beijing Solarbio Science & Technology Co., Ltd., (Beijing, China).

Animal model and treatment:

All experimental procedures were conducted in accordance with the guidelines of the institutional animal use and approved by the Ethics Committee of the North China University of Science and Technology. Specific Pathogen Free (SPF)-grade male Kunming mice (6-8 w old, weighing 18-22 g) were procured from Beijing Fukang Co., Ltd., (Beijing, China). Experimental Animal License Number is SYXK (Jing) 2020-0004. The mice were maintained in an environment at 22°±1° and exposed to 12 h photoperiod, followed by a period of darkness. 60 mice were divided into 6 groups: Control group (CON), SCOP group, Donepezil group (DON) and low, medium, and high-dose ICA groups (ICA-L, ICA-M, ICA-H)+SCOP. The CON and SCOP groups were given 0.5 % Calcium Magnesium Acetate (CMA)-Sodium (Na), the DON group was given 2 mg/kg DON hydrochloride, and the ICA-L, ICA-M, ICA-H groups were given 30 mg/kg, 60 mg/kg and 120 mg/kg ICA, respectively.

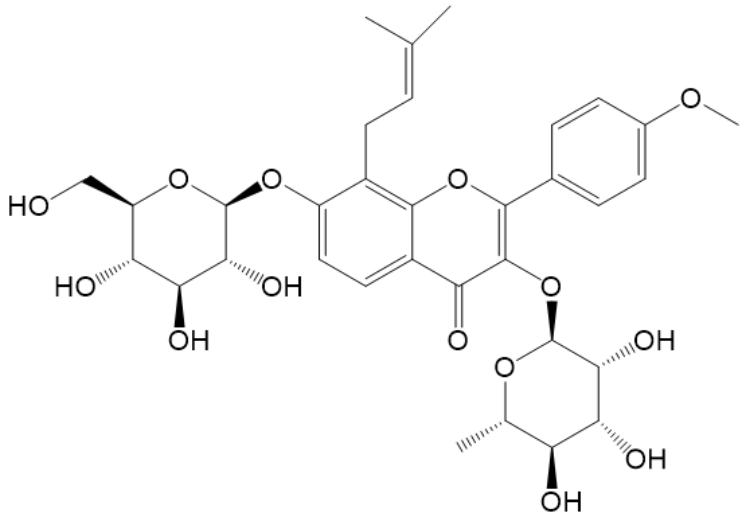

Fig. 1: Structural formula of ICA

All mice were given 10 ml/kg volume by continuous gavage for 14 d. Modeling was initiated on 10th d, the CON group was intraperitoneally injected with 0.9 % normal saline, and the other groups were intraperitoneally injected with 2 mg/kg SCOP hydrobromide (10 ml/kg) for 5 consecutive days to establish the cognitive impairment mouse model[14,15]. Behavioural experiments were conducted within 30 min of intraperitoneal injection.

Morris Water Maze (MWM) task:

The MWM task is a well-established test for assessing the learning and memory capabilities of animals[16]. Preliminary screening and training were performed prior to the test. The preliminary screening results showed that all mice had normal vision, and they could distinguish markers of different shapes and directions in the pool. Moreover, no difference was observed in their behavioral ability, and the mice could locate the platform. Therefore, individual differences in the mice had no influence on the results of behavioral experiments. From 10th d to 13th d, the positioning navigation experiment was performed, and the mice were placed in the pool to find the platform. The route map and time taken (escape latency) to find the platform were observed and recorded within 90 s. The spatial exploration experiment was carried out on 14th d, the platform was removed, and the memory of the mice was assessed by recording the times they crossed the original platform location within a 90 s timeframe[17].

New object recognition:

The new object recognition test is based on the curiosity of the mice towards novel objects[18]. A square box (50×50×40 cm^3) and 3 objects, A, B, and C, are used in the test. During the training phase, the mice were allowed to freely explore a box containing two identical objects A and B, and a different object C. The objects A and B were symmetrically placed in the box for 5 min. After a 24 h interval, object A was replaced with object C, and the test again performed. The time spent exploring the two distinct objects was recorded. The new object recognition index was calculated using the formula: (N-F)/(N+F)

where, N represents the time spent exploring object C, and F represents the time spent exploring object B.

Dark avoidance experiment:

Dark avoidance experiment is designed to detect the passive learning ability of animals[19]. The box is divided into two chambers, a bright chamber and a dark chamber (20×20×20 cm^3), with an opening hole between them, and in the experiment, the dark chamber had an electrified floor with a shock voltage set to 36 V. The mice were initially placed in a bright chamber facing a hole for 3 min facilitate their acclimatization to the surroundings. Then, the dark chamber was connected to the current. Because mice tend to move towards darkness, in this study, they were given an electric shock once they entered the dark chamber, following which they immediately returned to the bright chamber (this was recorded as an error). Each mouse was trained for 5 min. After 24 h, the memory retention ability of the mice was tested. The stay latency, which is the time spent by the mice to receive an electric shock, was measured. Additionally, the number of errors made within a 5 min period was recorded.

Biochemical index determination:

On the 14th d, after all behavioral experiments were completed, each mouse was given an intraperitoneal injection of 3 % sodium pentobarbital solution (10 ml/kg). After the mouse was decapitated under anesthesia, the brain tissue was quickly stored at -80°. Before analysis, the brains were homogenized to a 10 % brain tissue homogenate, and the supernatants were collected. The content of MDA, GSH, ACh and the activity of SOD, GSH-Px, AChE, ChAT were detected according to the manufacturer's instructions. These indicators were detected using an enzyme labeling instrument (molecular devices, USA) or spectrophotometer (PerkinsElmer, USA). The total protein was quantified using the Bradford assay[20].

Histological analysis:

On the 14th d, after the dark avoidance experiment, two mice from each group were given abdominal anesthesia with 3 % sodium pentobarbital solution. The chest was cut open to expose the heart, and normal saline was injected from the apex of the heart *via* a peristaltic pump. After most of the serum was cleared, 4 % paraformaldehyde solution was used for perfusion and fixation. After 24 h, the brains were embedded in paraffin and stained using Hematoxylin-Eosin (H&E)[21]. A light microscope (Olympus, Japan) was used to observe the neuronal morphology in the hippocampal Cornu Ammonis 1 (CA1) area.

Western blots analysis:

The brains were cut into pieces and lysed using Radioimmunoprecipitation Assay (RIPA) buffer, and the supernatants were collected[22]. The supernatants were added with 4 times loading buffer and then boiled at 100°. Following the assay[23], the protein was separated using a 10 %-15 % SDS-PAGE gel and then transferred onto Poly(Vinylidene Fluoride) (PVDF) membranes. The PVDF membranes were blocked in 5 % BSA for 2 h, and corresponding primary antibodies (BDNF 1:3000; PSD-95 1:3000; SYN 1:2500; caspase-3 1:1000; Bax 1:1000 and Barium Iodide (Bal-2) 1:1000) were added, following by incubation at 4° overnight. The following day, the membranes were washed and blocked again using a 1:10000 goat anti-rabbit antibody solution. The immunoblot bands were developed using an ECL solution, and a chemiluminescence imaging system was used for analysis[24].

Statistical analysis:

The experimental data were analysed using SPSS 25.0. Measurement data are expressed as the mean±Standard Deviation (SD). One-way Analysis of Variance (ANOVA) was performed to evaluate the significance of the two mean values. The difference was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Fig. 2, showed with the increase of training days, the escape latency of the SCOP group was gradually decreased, but there is still a significant gap with the CON group ($p<0.01$), indicating SCOP induced cognitive impairment in mice[25]. The DON group and all ICA groups were exhibited a significant reduction in the escape latency ($p<0.01$), indicating that treatment with DON hydrochloride and all doses of ICA may have a protective effect on the learning performance. In the space exploration experiment, the times of crossing the platform and swimming speed were decreased significantly in the SCOP group ($p<0.01$). However, the mice treated with DON hydrochloride and different doses of ICA showed an increased times of platform crossings and swimming speed ($p<0.05$).

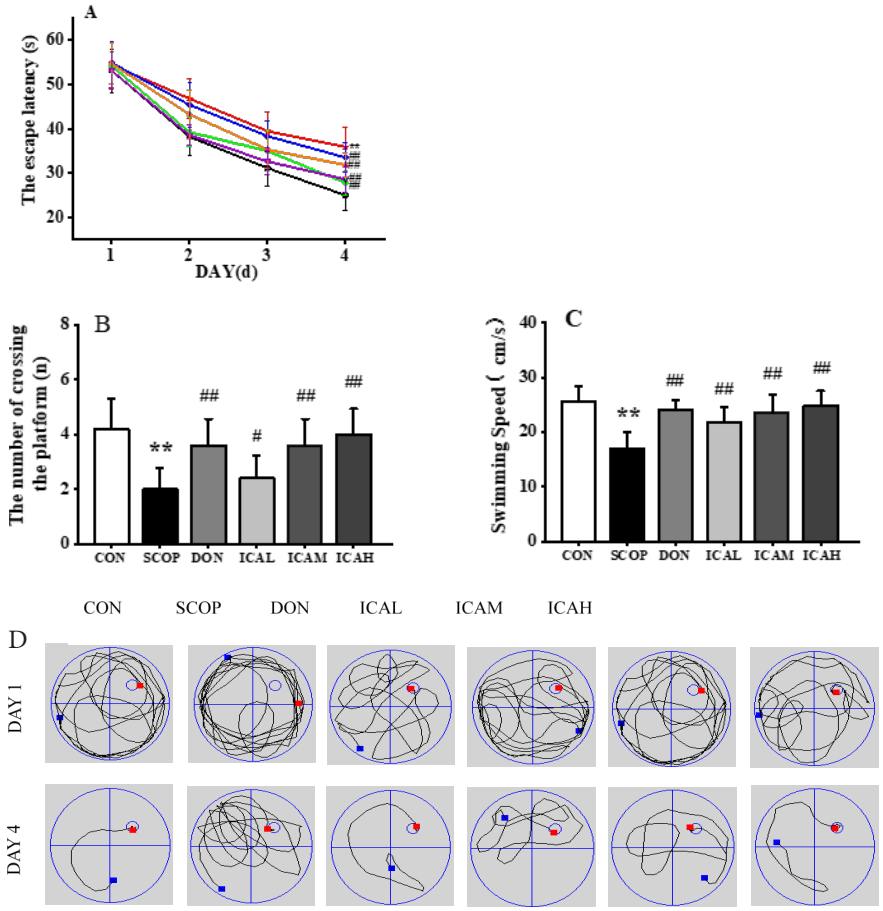

Fig. 2: A: Escape latency from 1st d to 4th d; B: The number of crossing platforms; C: Swimming speed in the space exploration experiment on the last day; D: Swimming trajectory on DAY 1 and DAY 4; (n=10)
Note: *p<0.05, **p<0.01 vs. CON group, #p<0.05, ##p<0.01 vs. SCOP group

According to fig. 3, the new object recognition index for the SCOP group was significantly reduced ($p<0.01$), indicating that the mice injected with SCOP were unable to distinguish between new and different objects. Conversely, the DON group and the ICA-M and ICA-H groups showed an increase in the new object recognition index ($p<0.01$), whereas the low-dose of ICA did not show any significant improvement trend. During the dark avoidance experiment, stay latency was found to decrease and the error times were found to increase significantly ($p<0.01$) in the SCOP group (fig. 4). These indicated that SCOP impaired the passive learning ability of the mice. After treatment with DON hydrochloride and medium-to-high doses of ICA, the stay latency improved significantly and error times reduced in various degrees ($p<0.01$).

The accumulation of lipid peroxides in the brain can lead to a decrease in antioxidant activity, which can result in an imbalance in the body's redox reaction and the production of a large amount of reactive oxygen species, leading to oxidative stress[26]. Oxidative stress can affect the normal physiological processes, causing systemic disorders and resulting in cognitive impairment[27]. As shown in fig. 5, the SCOP group exhibited a significant increase in MDA content and a decrease in SOD activity ($p<0.01$). However, after treatment with DON hydrochloride and different doses of ICA, the levels of MDA decreased and the activity of SOD increased significantly ($p<0.05$). Moreover, the SCOP group showed a significant decrease in the levels GSH-Px and GSH ($p<0.01$), which are major indicators of the body's antioxidant capacity and help maintain molecular physiological functions in the body[28], however, the levels of GSH-Px and GSH were increased after treatment ($p<0.01$). Previous studies have shown that ICA can regulate GSH content and GSH-Px activity[29], leading to an improvement in SCOP-induced neurobehavioral deficits. Therefore, enhancing the activity of antioxidant enzymes and reducing lipid peroxidation can alleviate cognitive impairment. These results indicated that ICA may reduce oxidative stress in mice and have promising therapeutic effects on the antioxidant system.

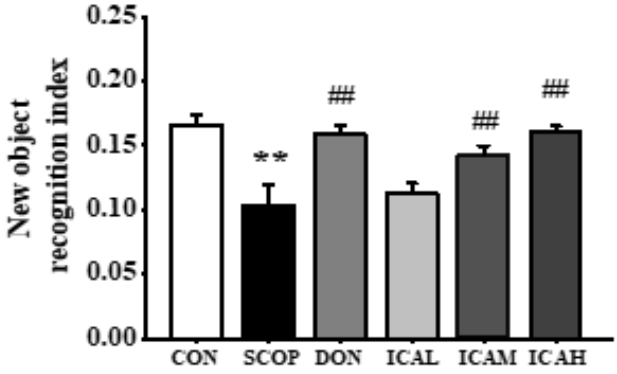

Fig. 3: The new object recognition index for the SCOP, DON, ICA-L, ICA-M and ICA-H groups (x̄±s, n=10)
Note: **p<0.01 *vs*. CON group and ##p<0.01 *vs*. SCOP group

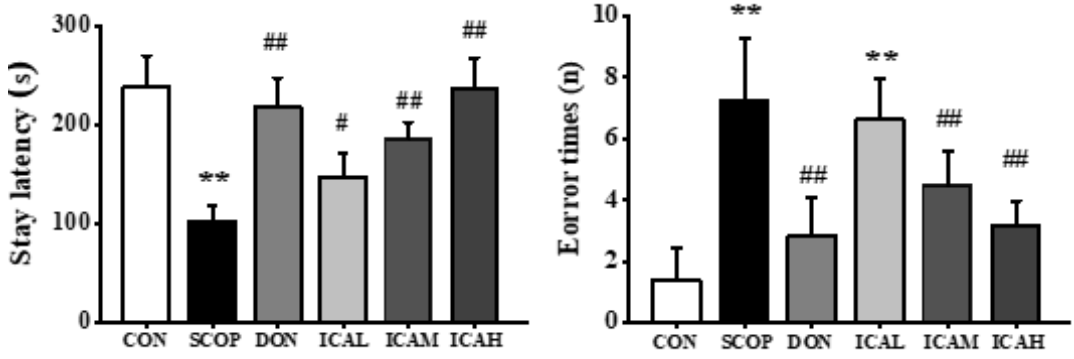

Fig. 4: Stay latency and error times of each group in the dark avoidance experiment (x̄±s, n=10)
Note: **p<0.01, #p<0.05 *vs*. CON group and ##p<0.01 *vs*. SCOP group

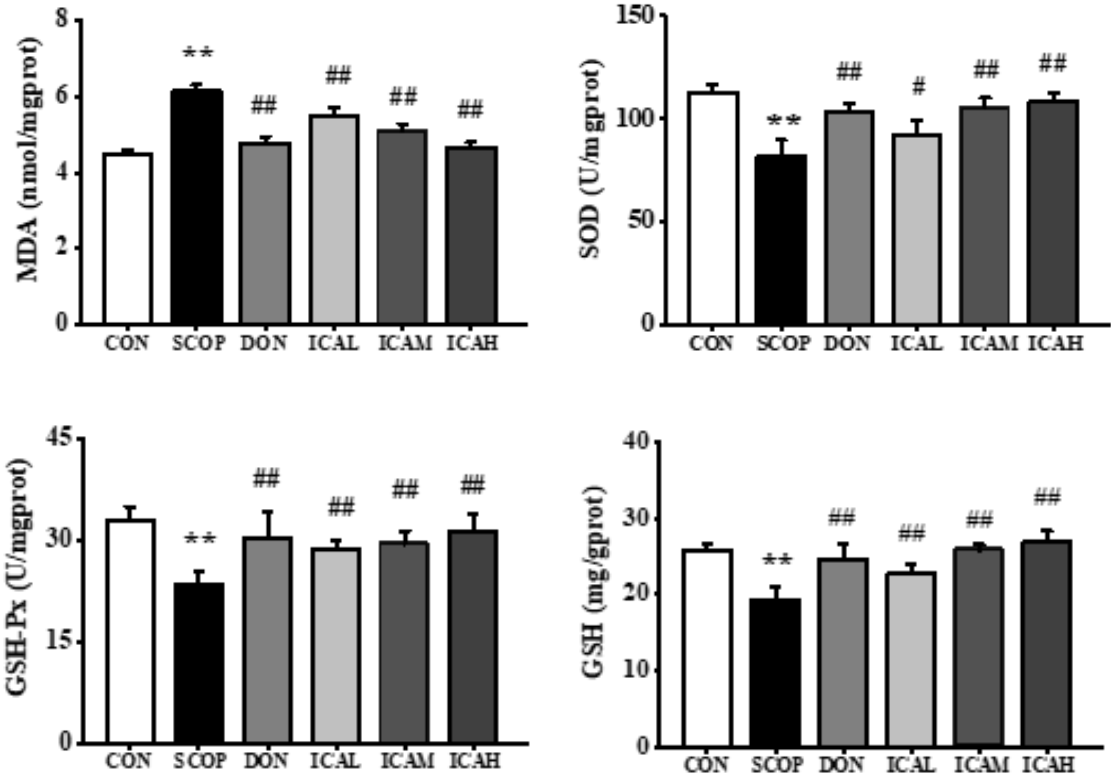

Fig. 5: Effects of ICA on oxidative stress in the mice with SCOP-induced cognitive impairment (x̄±s, n=8)
Note: **p<0.01 *vs.* CON group, #p<0.05 and ##p<0.01 *vs.* SCOP group

Cholinergic impairment is a significant contributor to cognitive impairment. Recent studies[30] have indicated that most individuals with cognitive impairment exhibit a lack of synthesis and release of ACh while its activity of AChE and ChAT, which leads to serious functional degeneration of the cholinergic system and eventually affecting the learning and memory ability. ACh, an important neurotransmitter, contributes to information transmission and cognitive processes. The synthesis and degradation of ACh are closely related to ChAT and AChE. The main functions of AChE are to terminate synaptic transmission, inhibit the excitatory action of neurotransmitters, and thus affect the function of cholinergic system. However, ChAT promotes the generation of ACh. Fig.6, shows in the SCOP group, the activity of AChE was increased, but the levels of ChAT and ACh were decreased significantly ($p<0.01$), indicating that the cholinergic system was damaged, which contributed to cognitive impairment. After the treatment with donepezil hydrochloride and corresponding doses of ICA, the levels of ChAT and ACh increased, and the activity of AChE decreased significantly ($p<0.05$). Current research[31] has shown that schisanhenol could inhibit AChE activity, and promote ACh generation, thereby improving the cholinergic system, and alleviating cognitive impairment. Similarly, the present study showed that ICA had the ability to restore ACh and ChAT levels, reduce AChE activity, and protect the cholinergic system.

The hippocampus, located on the medial side of the temporal lobe, plays crucial roles in memory retention, learning, and building a sense of space. CA1 is located in the dorsal part of the hippocampus, and hippocampal CA1 neurons participate in the primary coding and processing of spatial information[32]. If the neurons are stimulated by external or internal boundaries, the number of cells will decrease with the apoptosis, leading to the decline of spatial memory[33]. Microscopic observation results (fig.7), showed that in the CON group, the hippocampal CA1 area's structure was normal, with closely arranged neurons, large and regular cells with 4-6 layers, a round nucleus, and a distinct nucleolus. However, the SCOP group exhibited severe pathological changes in the CA1 area, with disordered neuronal arrangement and cell death, resulting in a loose area, irregular-shaped nuclei, and fuzzy nucleoli. In the ICA-L group, the pathological changes were not significant, and the neuronal arrangement was closer than that of the SCOP group, although the cell status was poor. In the ICA-M group, neuronal morphology

was improved, the arrangement of neurons was orderly and compact, and the pathological state was alleviated. In the DON and ICA-H groups, neuronal morphology was significantly improved, with a neat and tight neuronal arrangement and improved cytopathy. These results indicated that ICA and DON hydrochloride significantly alleviated the hippocampal neuron morphology in the mice with cognitive impairment.

Synaptic proteins are linked to individuals with neurological disorde[34]. BDNF, PSD-95 and SYN are closely related to synaptic plasticity, and the interaction of these proteins with membrane nerve-related guanylate kinase is crucial for synaptic transmission across neurons. Research has showed that ICA can increase the levels of BDNF and TrkB in the hippocampus of rats with D-gal-induced cognitive impairment[35], and alleviate memory loss in mice. Additionally, after several months of ICA treatment for cognitive impairment in mice, the expression of PSD-95 was found to significantly increase[36]. Consistent with these findings, in this study, that the expressions of BDNF, PSD-95 and SYN were decreased significantly in the SCOP group ($p<0.01$) as shown in fig. 8. However, in the mice treated with DON hydrochloride and different doses of ICA, the expressions of BDNF, PSD-95 and SYN increased significantly ($p<0.05$). Moreover, the ICA dose of 30 mg/kg was effective in increasing SYN expression but not PSD-95 expression. It was observed that ICA could increase the levels of BDNF, PSD-95 and SYN significantly, which helped restore synaptic plasticity (a key link in the process of memory formation), alleviated the loss or dysfunction of synaptic proteins, and reduce cognitive impairment. Overall, the study provides preliminary evidence supporting the potential therapeutic role of ICA in cognitive impairment (fig. 9).

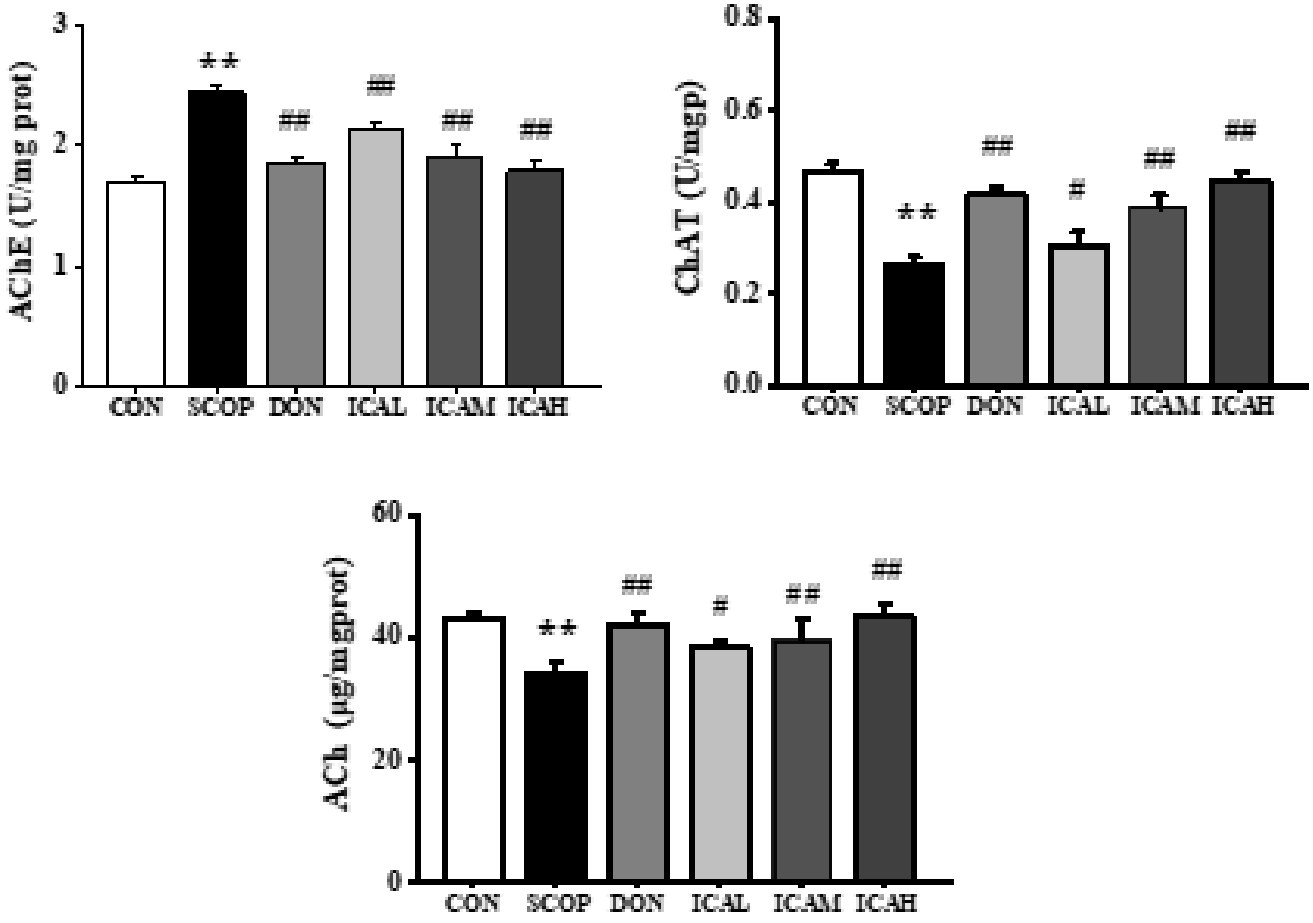

Fig. 6: Effect of ICA on the activity of AChE and ChAT, and the content of ACh (x̄±s, n=8). **p<0.01 *vs*. CON group; #p<0.05 and ##p<0.01 *vs*. SCOP group

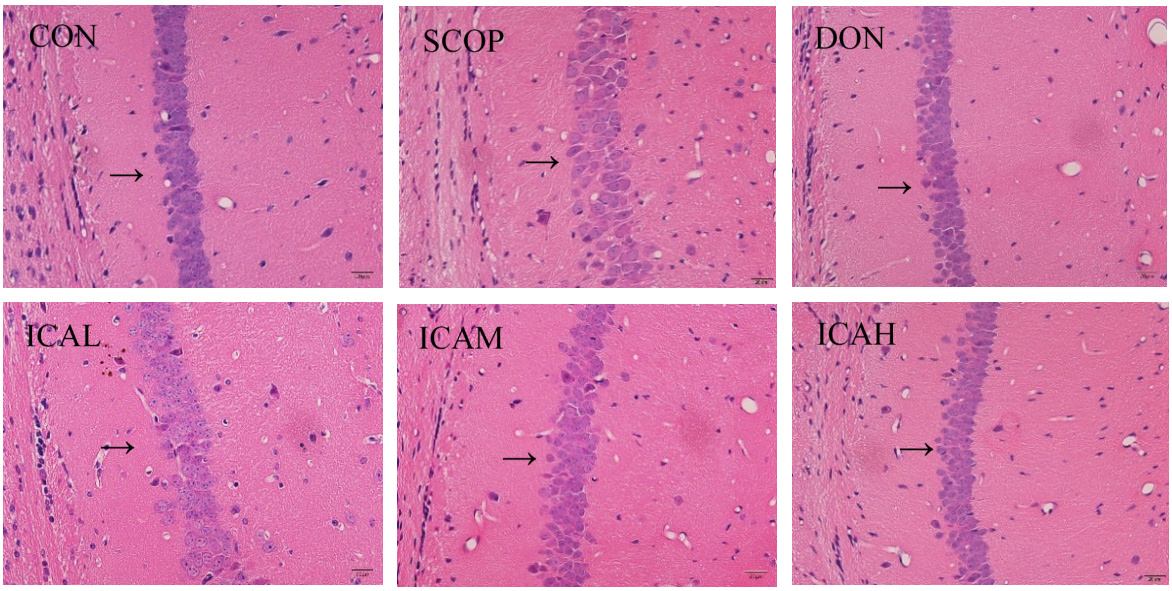

Fig. 7: Effect of ICA on neurons in the hippocampal CA1 region of the mice. The brains were embedded in paraffin and subjected to H&E staining; the degree of CA1 damage was observed in 10 random fields per animal at 400X magnification

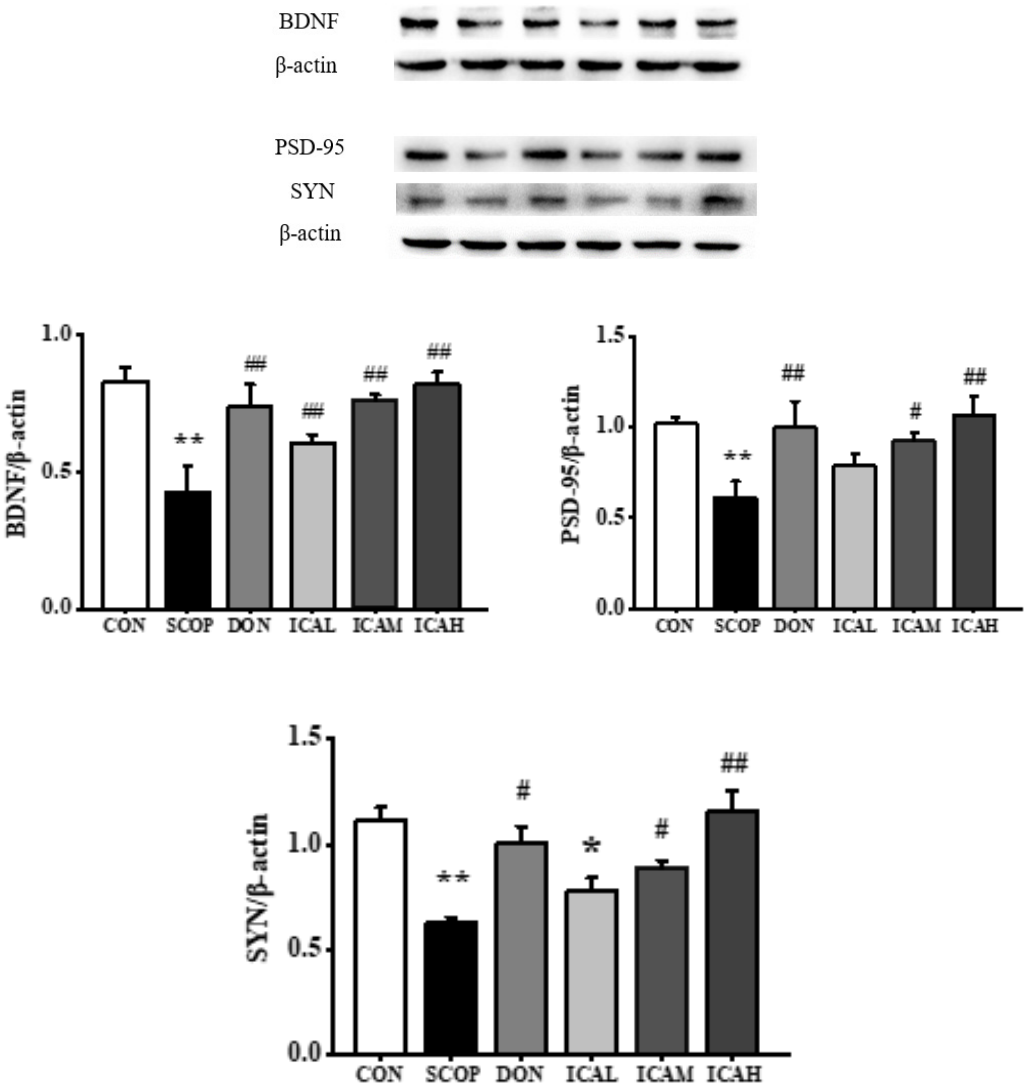

Fig. 8: ICA increased the expression of proteins BDNF, PSD-95 and SYN ($\bar{x}$±s, n=6)
Note: **p<0.01 *vs.* CON group and ##p<0.01 *vs.* SCOP group

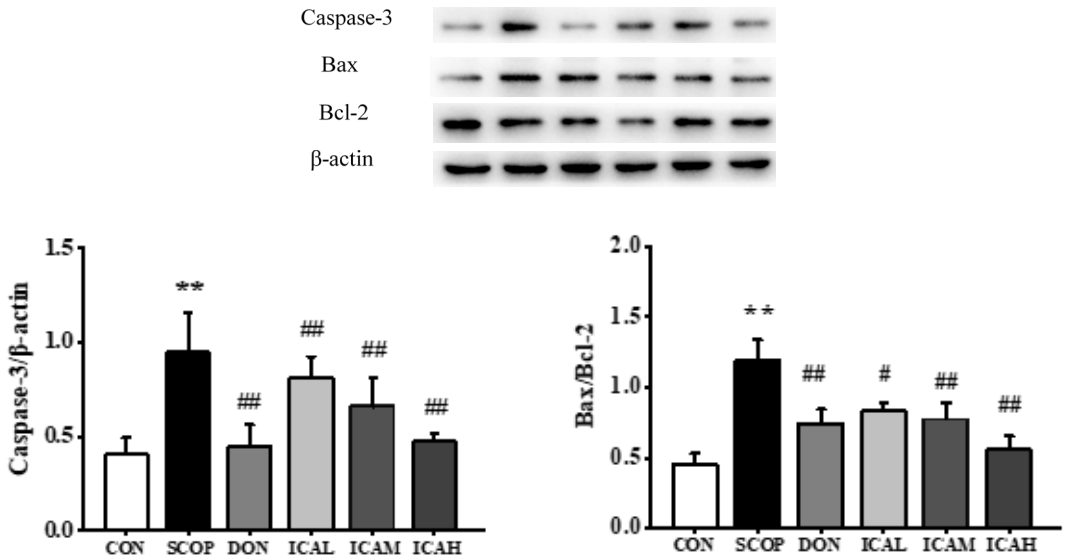

Fig. 9: ICA inhibited the expression of caspase-3 and reduced the Bax/Bcl-2 ratio ($\bar{x}$±s, n=6)
Note: **p<0.01 *vs*. CON group, #p<0.05 and ##p<0.01 *vs*. SCOP group

Caspase-3 is a key protease that promotes apoptosis, in addition to hydrolysing target substances in cells and degrading proteins in cells[37]. The Bax/Bcl-2 ratio is a key indicator of apoptosis, with high ratios indicating an increased susceptibility to apoptosis[38]. Research has demonstrated the potential role of ICA in decreasing cleavage of caspase-3, caspase-9, and caspase-12 in the hippocampal tissue, indicating that ICA can help maintain the number of neurons in the brain of AD mice and reduce cell apoptosis[39,40]. In this study, the intraperitoneal injection of SCOP promoted cell apoptosis in the SCOP group, resulting in a remarkable increase in caspase-3 expression and the Bax/Bcl-2 ratio ($p<0.01$). However, after the treatment with DON hydrochloride and ICA, the expression of caspase-3 decreased significantly. Furthermore, the administration of DON and different doses of ICA decreased the Bax/Bcl-2 ratio, which suggests its protective effect against apoptosis. These findings indicate that ICA may exert a protective effect against SCOP-induced cell apoptosis by modulating the expression of apoptotic markers such as caspase-3 and Bax/Bcl-2 ratio. Academic community has paid considerable attention to the issue of cognitive impairment, however, its specific pathogenesis remains unclear. Oxidative stress, cholinergic system impairment, apoptosis, and other phenomena have been reported in the brain of individuals with cognitive impairment[41,42]. SCOP, an M receptor antagonist, can prevent ACh from binding to M receptor. It blocks the transmission of information and thus interferes with the formation of memory[43], finally leading to cognitive impairment. It is similar to the typical cognitive impairment model. DON is a widely used therapeutic drug for mild and moderate cognitive impairment[44], therefore it is used as the positive CON. ICA, which is an effective component of traditional Chinese medicine *Epimedium brevicornum* Maxim, has various pharmacological activities such as antioxidant and anti-inflammatory properties. Moreover, ICA has displayed a strong reducing ability and exhibits strong ability to scavenge free radicals. Different doses of ICA have been found to improve the learning and memory abilities of Senescence-Accelerated Mouse Prone 10 (SAMP10), and its mechanism of action was related to the cholinergic system[45]. Studies have also shown that ICA can improve vascular dementia by regulating the expressions of BDNF and TrkB[46]. These studies indicate that the therapeutic effect of ICA on cognitive impairment is promising. In the present study, ICA improved the learning performance in a SCOP-induced cognitive impairment model, which was validated from multiple aspects and targets. In conclusion, this study suggests that ICA can alleviate cognitive impairment possibly by upregulating the expression of BDNF, PSD-95, and SYN-related synaptic proteins, and inhibiting caspase-3 expression to reduce apoptosis and restore normal synaptic function. ICA can also protect the hippocampal neurons from oxidative stress and maintain their morphological function, while restoring the cholinergic system.

Acknowledgments:

This work was supported by the Natural Science Foundation of Hebei Province, China (Grant Number H2021209059) and Project on Doctoral Research Start-up Foundation of North China University of Science and Technology, China (Grant Number 25759799).

Conflict of interests:

The authors declared no conflict of interests.

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