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Protective effects of isofraxidin against scopolamine-induced cognitive and memory impairments in mice involve modulation of the BDNF-CREB-ERK signaling pathway

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Abstract

Background: Isofraxidin is a coumarin compound mainly isolated from several traditional and functional edible plants beneficial for neurodegenerative diseases, including *Sarcandra glabra* and *Apium graveolens*, and *Siberian Ginseng*.

Objective: This study aimed to assess effects of isofraxidin against memory impairments and cognition deficits in a scopolamine-induced mouse model.

Materials & methods: Animals were randomly divided into 6 groups, control, vehicle, donepezil (10 mg/kg, p.o.), and isofraxidin (3, 10, and 30 mg/kg, p.o.). Isofraxidin or donepezil was administered for 44 days, once per day. The scopolamine insults (1 mg/kg, i.p.) was given from the 21st day, once per day. Morris water maze test and Y-maze test were used for the behavioral test. After that, brain samples were collected for analysis.

Results: Firstly, isofraxidin significantly improved scopolamine-induced behavioral impairments and cognition deficits in Morris water maze and Y-maze test. Then, isofraxidin facilitated cholinergic activity via inhibiting acetylcholinesterase (AChE) activity. Besides, isofraxidin decreased lipid peroxidation level but enhanced levels of glutathione, glutathione peroxidase, and superoxide dismutase. Moreover, isofraxidin suppressed the expression of inflammatory mediators and cytokines. Further investigations showed that isofraxidin up-regulated expression of brain-derived neurotrophic factor (BDNF), and promoted phosphorylation of tropomyosin-related kinase B (TrkB), cyclic AMP-response element-binding protein (CREB), and extracellular signal-regulated kinase (ERK).

Discussion & Conclusion: These results suggested that isofraxidin ameliorated scopolamine-induced cognitive and memory impairments, possibly through regulating AChE activity, suppressing oxidative stress and inflammatory response, and modulating BDNF-CREB-ERK pathways.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and has been considered to be one of the global health concerns(Weinreb et al. 2016). The clinical manifestations of AD are usually characterized by memory impairments and cognition deficits(Li et al. 2016). The occurrence and development of AD involve several pathogenic mechanisms, including cholinergic dysfunction, oxidative stress, inflammatory response, amyloid plaques generation, and neurofibrillary tangles formation(Fabiani et al. 2018; Wojsiat et al. 2018). Currently, the most preferentially prescribed therapy is acetylcholinesterase (AChE) inhibitors via alleviating cholinergic neurotransmitter system dysfunction(Fabiani et al. 2018; Wojsiat et al. 2018). Besides, anti-oxidative and anti-inflammatory drugs are also applied as adjunctive treatments for AD. However, there exist several limitations in the current agents, including inefficiencies, short half-lives, and apparent adverse effects. Thus, it is still necessary to find out more safe and effective agents for AD. In this respect, complementary and alternative

approaches like natural products and dietary supplements have been proved to be preventative and therapeutic for AD.

Scopolamine is a kind of competitive antagonist of muscarinic acetylcholine receptors (AChRs) that could modulate the cholinergic transmissions in the central nervous system (CNS)(Ahmad et al. 2014; Rabiei and Setorki 2018; Hoang et al. 2020). Systemic administration of scopolamine in rodent animals could lead to AD-like characteristic symptoms, like neuron loss, cholinergic deficiency, memory impairment, and cognition dysfunction(Ahmad et al. 2014; Rabiei and Setorki 2018; Hoang et al. 2020). Hence, scopolamine-induced experimental models have been widely used for screening potential memory and cognition-enhancing agents(Ahmad et al. 2014; Rabiei and Setorki 2018; Hoang et al. 2020). Accumulating evidence indicates that scopolamine insults not only induce cholinergic system dysregulations but also result in excessive oxidative stress and neuroinflammatory damages(Ahmad et al. 2014; Rabiei and Setorki 2018; Hoang et al. 2020). Besides, the scopolamine insults also result in the dysfunction in modulatory components regulating synaptic plasticity and cognitive activity, like brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB), cAMP-response element-binding protein (CREB), and extracellular signal-regulated kinase (ERK) in various kinds of animal models(Tyler et al. 2002). Notedly, accumulating reports indicate that agents targeting the BDNF-CREB-ERK pathways could always confer protective actions against neurodegenerative diseases *in vitro* and *in vivo*(Hafez et al. 2017; Ko et al. 2018). Therefore, at present, oxidative stress, neuroinflammation, and BDNF-CREB-ERK signaling are promising targets for developing novel anti-AD agents.

Isofraxidin, (7-hydroxy-6,8-dimethoxycoumarin, Fig. 1A), is a coumarin compound which mainly exists in several traditional and functional edible plants in China, like *Sarcandra glabra* and *Apium graveolens*, and *Siberian Ginseng*. These plants have been widely used as traditional herbal medicine to provide beneficial actions against neurodegenerative diseases(Huang et al. 2012; Niu et al. 2015). Previous reports have indicated that isofraxidin confers considerable anti-oxidative and anti-inflammatory properties(Huang et al. 2012; Niu et al. 2015). It can protect against radiation-induced apoptosis and death in human leukemia cells via regulating reactive oxygen species (ROS)-related pathways(Li P et al. 2014). Accumulating evidence also indicates that isofraxidin can alleviate inflammatory damages in lipopolysaccharide (LPS)-induced acute lung injury in mice and high-fat-diet-induced hepatic lipid homeostasis *in vivo*(Li J et al. 2017). Also, the inhibitory actions of isofraxidin on AChE activity *in vitro* are indicated in the previous report(Polatoglu et al. 2017). The isofraxidin-mediated effects on AChE activity, oxidative stress, and inflammation indicate its potential for AD prevention and treatment(Deng et al. 2020). Thus, this study aimed to investigate the effects of isofraxidin against scopolamine-induced behavioral impairments in mice. Finally, the role of isofraxidin in AChE activity, oxidative stress, inflammatory response, and BDNF-CREB-ERK pathways were also further investigated.

Materials And Methods

Materials and chemicals

Isofraxidin (power, purity: UV ≥ 98%) was obtained from Weikeqi Biological Technology Co. Ltd. (Sichuan, China). Donepezil hydrochloride monohydrate was purchased from J&K Scientific Co. Ltd. (Beijing, China). The carboxymethyl cellulose sodium salt (CMC-Na) and scopolamine were obtained from Sigma-Aldrich (MO, USA).

Animals and experimental groups

The C57BL/6 mice (male, about 8 weeks old, 20 to 25 g) were supplied by the Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, No. 44007200085200) and maintained in SPF environments. Animals were maintained on 12-hour light/12-hour dark cycle, under standard conditions, with regular daily diets and clean water *ad libitum*. No animal enrichments were given in our study. The procedures and protocols were approved by the Laboratory Animal Ethics Committee of Guangzhou Medical University (GY2020-055), and animal experiments were perferemced in the Laboratory Animal Centre Institutional Animal Care and Use Committee, Guangzhou Medical University.

The experimental schedule was shown in Fig. 1B. Donepezil is a specific and reversible inhibitor of AChE. It has been used for the symptomatic treatment of mild to moderate AD clinically, and is also widely used in many AD animals' models. Therefore, donepezil was used as a positive control agent in this study. After a 7-day adaptation, the total amount of 54 mice were then randomly divided into 6 groups (9 animals in each group; dividing into two cages, 4 or 5 per cage): control (saline + vehicle), vehicle (scopolamine + vehicle), isofraxidin 3 (scopolamine + 3 mg/kg isofraxidin), isofraxidin 10 (scopolamine + 10 mg/kg isofraxidin), and isofraxidin 30 (scopolamine + 30 mg/kg isofraxidin), donepezil 10 (scopolamine + 10 mg/kg donepezil). A series of isofraxidin solution (0.3, 1 and 3 mg/mL) were prepared by dissolving isofraxidin into the CMC-Na-water solution (0.5%, *w/v*). The isofraxidin solution was freshly prepared and orally administrated (0.1 mL/10g animal weight) to mice daily for 3 weeks in advance. Scopolamine insults from the 21st day were used according to previous studies[5]. Briefly, scopolamine was prepared in saline and was given (1 mg/kg, i.p.) 60 min after various drug treatments. All animals in each were used in behavioral tests, including the Morris water maze test and Y-maze test, were performed 30 min after the scopolamine injection.

Behavioral tests

Morris water maze test

The Morris water maze test was performed according to previous publications with small modifications(Morris 1984; Wu et al. 2018) using the Morris Water Maze video analysis system (Beijing ZS Dichuang Technology Development, Beijing, China). The circular pool (diameter, 120 cm; height, 60 cm) was equally divided into quadrants, and on the wall of each quadrant, the distinct color sign was marked as a visual positional hint. The water was kept at 22 ± 1°C and rendered opaque by food-grade titanium dioxide. A platform (diameter 8 cm; height 30 cm) was positioned inside the tank with its top submerged 1 cm below the water surface in the target quadrant of the maze. The video tracking camera

was mounted onto the ceiling directly above the center of the pool to record and detect swimming parameters.

24 hours before the Morris water maze test, all animals were habituated to the pool environment for 120 s without the platform. The Morris water maze experiment consists of two parts: navigation trials and spatial probe trials. In the navigation trials, mice were trained for 4 days with 4 trials per day (interval of 15 min) and were assigned four pseudorandom starting points each day. During each trial, mice were placed into the water facing the wall of the pool at the 1/2 radian position in one of the four quadrants and allowed to locate the submerged platform. A successful trial was recorded when a mouse found the platform within 60 s. Then the animal was allowed to stay on the platform for 5 s before being returned to its home cage. If a mouse was failed to locate the platform within 60 s, it was gently guided to the platform and allowed to stay on it for 15 s. The time required (escape latency) to find the escape platform and the swimming speed were measured using the video tracking software. The maximum value for escape latency was set at 60 s. The spatial probe trial was performed on the 5th day. During this trial, the platform was removed from the pool, and mice were placed into the water facing the pool wall. Mice were allowed to swim freely for 60 s. The number of times mice crossing the original platform location, as well as total swimming time and distance in the original platform quadrant, was recorded.

Y-maze test

The Y-maze apparatus consists of 3 equal arms (35 cm long, 5 cm wide, and 10 cm height) and a video tracking system (Beijing ZS Dichuang Technology Development)(Conrad et al. 1996). Arms that are made of white polyvinyl chloride are positioned at an equal angle of 120° from each other and extend from the central platform. Arms are labeled with A, B, and C, and on the inner walls of each arm, distinct colored signs were marked as visual positional hints. The video tracking camera was mounted onto the ceiling directly above the center of the pool to monitor the tracks of the mice.

Spatial novelty recognition. During the training trial, one arm (C) was closed, and the animals were positioned in one of the open arms (A) and allowed 15 min to explore the open arms (A and B) of the Y-maze(Ennaceur and Delacour 1988; Hughes 2004). All mice were returned to their home cages and given an hour inter-trial interval before commencing the next phase. During the test trial, mice were allowed to explore the maze for 5 min with all arms open, and the time spent in the novel arm (C) was recorded. All arms were cleaned with ethanol solution between each trial to avoid olfactory clues.

Spontaneous alternation. To measure spatial memory, the animal was placed in the center of the maze and allowed to explore the maze freely for 6 min. The arm entry sequence was recorded. A successful alteration is represented by the animal visiting all three arms consecutively (ABC, ACB, BCA, BAC, CAB, and CBA)(Ennaceur and Delacour 1988; Hughes 2004). The percentage of alternation was calculated according to the formula: spontaneous alternation/total possible alternations × 100. All arms were cleaned with ethanol solution between each trial to avoid olfactory clues.

Brain sample collection and preparation

After the animal behavioral test, mice were sacrificed using carbon dioxide inhalation. Then the brain was harvested and dissected immediately. The cerebral cortex and hippocampus were separated on ice immediately. All samples were then kept at -80°C until assay.

Determination of AChE activity and ACh level

The AChE activity was measured using an acetylcholinesterase activity colorimetric assay kit (K764, BioVision, CA, USA) according to the manufacturer's protocols. In brief, the samples were homogenized after adding cold lysis buffer in the ice. The samples were then centrifuged for 10 min at 12000 rpm. The supernatant was then collected and subjected to further assay. The ACh level was measured using the acetylcholine ELISA kit (E4453, BioVision) according to the manufacturer's protocols. Briefly, the samples were homogenized after adding cold PBS (pH 7.4) in the ice. The samples were then centrifuged for 20 min at 3000 rpm. The supernatant was then collected and subjected to further assay.

Assessment of lipid peroxidation (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)

The levels of lipid peroxidation, GSH, GSH-Px, and SOD were measured using MDA (S0131), GSH (S0053), GSH-Px (S0056), and SOD (S0109) assay kits (Beyotime, Shanghai, China), according to manufacturer's protocols, respectively. In brief, the samples were homogenized after adding cold lysis buffer in the ice. The samples were then centrifuged for 10 min at 12000 rpm. The supernatant was then collected and subjected to further assay.

Western blotting assay

Aliquots protein samples (about 40 μg) were resolved by SDS-PAGE (7.5–12%) and transferred to PVDF membranes (Bio-Rad, CA, USA). The blots were then incubated with appropriate primary antibodies, including BDNF (AB1534, 1:1000, Millipore), p-TrkB (ABN1381, 1:500, Millipore), iNOS (#2982, 1:1000, CST), COX-2 (#12282, 1:1000, CST), TrkB (#4603, 1:1000, CST), p-CREB (#9198, 1:1000, CST), t-CREB (#9197, 1:1000, CST), p-ERK (#4377, 1:1000, CST), t-ERK (#4695, 1:1000, CST), β-tubulin (#2128, 1:2000, CST), and β-actin (#4970, 1:2000, CST), and peroxidase-conjugated secondary antibodies (CST), respectively. Finally, protein bands were visualized using an ECL plus Western blotting detection reagents (GE Healthcare). The membranes were then scanned on a Bio-Rad Chemi Doc XRS Imaging System, and the intensity of the protein bands was analyzed using Bio-Rad Quantity One Software (Bio-Rad).

Graphing and statistical analysis

Statistical analyses were performed using GraphPad Prism software (ver. 6.0; GraphPad, CA, USA), and data are represented as means ± standard (SD). Repeated-measures one-way variance (ANOVA) or one-way ANOVA followed by Newman-Keuls *post hoc test* were data analysis. $P < 0.05$ was considered significant in all analyses.

Results

Isofraxidin ameliorates scopolamine-induced memory and cognitive impairment

In the Morris water maze test, spatial learning, memory, and cognition can be assessed by time to reach the platform (escape latency), time spent in target quadrant, and platform crossings, respectively (Wu et al. 2018). Results indicated that isofraxidin treatments (10 and 30 mg/kg) could significantly reduce the escape latency on day 3 and day 4 when compared to the vehicle group (Fig. 2A, repeated-measures one-way ANOVA test, day 3, $F(5, 48) = 10.83$, $p < 0.0001$, and compared with the vehicle group, $p < 0.05$ at 10 mg/kg, $p < 0.01$ at 30 mg/kg; day 4, $F(5, 48) = 9.606$, $p < 0.0001$, compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg). During the probe test on the 5th day, time spent in the target quadrant and platform crossing were then calculated. As shown in Fig. 2B and 2C, animals treated with isofraxidin (10 and 30 mg/kg) showed significant increases in the time spent in the target quadrant ($F(5, 48) = 11.76$, $p < 0.0001$, and compared with the vehicle group, $p < 0.01$ at 10 and 30 mg/kg) and platform crossings ($F(5, 48) = 18.19$, $p < 0.0001$, and compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg), respectively. Next, these results were further confirmed by the Y-maze test. As shown in Fig. 3A, isofraxidin-treated animals could spend more time in the novel arm ($F(5, 48) = 30.75$, $p < 0.0001$, and compared with vehicle, $p < 0.05$ at 3 mg/kg, $p < 0.01$ at 10 and 30 mg/kg) compared to vehicle group. Besides, the percentage alterations were also significantly increased (Fig. 3B, $F(5, 48) = 19.42$, $p < 0.0001$, and compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg) in isofraxidin-treated groups compared to the vehicle group. The mice from positive control (donepezil treatment) also exerted significant amelioration in cognitive deficits in Morris water maze and Y-maze tasks (compared with vehicle, $p < 0.01$).

Isofraxidin restores the scopolamine-induced dysfunction in cholinergic activity

To explore the action of isofraxidin on cerebral cholinergic transmission, indicators of cholinergic activity, including AchE activity and Ach level, were also evaluated, respectively. As shown in Fig. 4A, the AChE activity was increased in the hippocampus with scopolamine insult. However, isofraxidin treatments could significantly decrease the levels of AChE activity (compared with vehicle, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg) compared to vehicle mice, in a dose-dependent manner. Additionally, the scopolamine-treated group showed a significant decrease in ACh level in the hippocampus (Fig. 4B, ompared with control, $p < 0.01$). However, isofraxidin treatments could significantly restore scopolamine-induced decreases in ACh (Fig. 4B, compared with vehicle, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg). Besides, donepezil treatment also significantly decreased the AChE activity and increased the Ach level in the hippocampus (compared with vehicle, $p < 0.01$).

Isofraxidin suppresses scopolamine-induced oxidative stress

To investigate the protective effects of isofraxidin on scopolamine-induced oxidative stress, MDA, GSH, GSH-Px, and SOD levels in the hippocampus were then measured. The level of MDA in the hippocampus was elevated significantly following the scopolamine treatment (Fig. 5A, compared with the control group, $p < 0.01$), whereas isofraxidin significantly revised this tendency (compared with the vehicle group, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg). The declined SOD activity was observed in the scopolamine-treated group, but isofraxidin treatment significantly enhanced the SOD activity (Fig. 5B, compared with vehicle, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg). Also, isofraxidin could improve the GSH-Px activity in the hippocampus of scopolamine-treated mice (Fig. 5C, compared with vehicle, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg). Also, isofraxidin significantly increased the GSH levels in the hippocampus in scopolamine-treated mice (Fig. 5D, compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg). Notedly, donepezil treatment also significantly increased the level of GSH, GSH-Px, and SOD, but decreased the MDA production in the hippocampus in scopolamine-treated mice (compared with vehicle, $p < 0.01$). In summary, these results suggested that isofraxidin may prevent scopolamine-induced oxidative damages in the brain.

Isofraxidin alleviates scopolamine-induced inflammatory response

Then, we investigated isofraxidin actions on the expression of inflammatory mediators and cytokines in scopolamine-induced mice hippocampus. As shown in Fig. 6A and 6C, the scopolamine treatment induced an increase in the NO and PGE_2 levels in the hippocampus. However, isofraxidin significantly blocked the NO production (compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg) and PGE_2 (compared with vehicle, $p < 0.05$ at 10 mg/kg and $p < 0.01$ at 30 mg/kg). It is well reported that iNOS and COX-2 modulate the expression of NO and PGE_2, respectively. Notedly, as shown in Fig. 6B and 6D, we found that isofraxidin treatments could significantly inhibit the scopolamine-induced increase in the expression of iNOS and COX-2 (iNOS, compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg; COX-2, compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg). Moreover, the inhibitory actions of isofraxidin on the TNF-α and IL-1β production were also observed. As shown in Fig. 6E and 6F, isofraxidin-treatment could significantly decrease the TNF-α (compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg) and IL-1β level (compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg) in the hippocampus. Additionally, donepezil treatment also significantly decreased the level of NO, PGE_2, iNOS, and COX-2 in the hippocampus in scopolamine-treated mice (compared with the vehicle group, all $p < 0.01$). These results suggested that isofraxidin might alleviate scopolamine-induced inflammatory response and damage in the brain.

Isofraxidin up-regulates BDNF expression and promotes phosphorylation of TrkB, ERK, and CREB

To further explorer the mechanisms involved in isofraxidin-mediated protection against scopolamine-induced memory impairment, we next detected isofraxidin role on several components that modulate synaptic plasticity and cognitive activity. As shown in Fig. 7A, scopolamine significantly decreased the

BNDF expression in the hippocampus. However, isofraxidin restored this scopolamine-induced decrease in BNDF (compared with vehicle, $p < 0.01$ at 10, and 30 mg/kg). Meanwhile, we also found that isofraxidin could significantly promote a scopolamine-induced decrease in phosphorylation of TrkB (Fig. 7B, compared with vehicle, $p < 0.01$ at 10 and 30 mg/kg). Besides, we also found that scopolamine significantly decreased phosphorylated levels of CREB (Fig. 7A and 7C, compared with control, $p < 0.01$) and ERK in the hippocampus (Fig. 7A and 7D, compared with control, $p < 0.01$). However, isofraxidin restored and promoted phosphorylation of CREB (compared with vehicle, $p < 0.01$ at 10, and 30 mg/kg) and ERK (compared with vehicle, $p < 0.01$ at 10, and 30 mg/kg) in the scopolamine-treated hippocampus.

Discussion

Nowadays, accumulating reports indicate the potential of naturally occurring coumarins and flavonoids in the modulation of neuronal functions in neurodegenerative diseases(Li C et al. 2018). Isofraxidin is dietary coumarins that have been disclosed to confer inhibitory actions on AChE activity, oxidative stress, and inflammation, which indicated it might provide a regime for AD preventions and treatments. Hence, in this study, the neuroprotective action of isofraxidin against scopolamine-induced memory impairment and cognition deficits in mice were investigated.

Firstly, the memory and cognitive function were assessed using the Morris water maze test and Y-maze test(Ahmad et al. 2014; Rabiei and Setorki 2018; Hoang et al. 2020). The Morris water maze test is dependent on hippocampal functions and is commonly used to assess spatial learning and long-term memory via measurement of escape latency during the training sessions and swimming times and crossing numbers within target quadrants during spatial probe trials(Bajo et al. 2015; Hescham et al. 2009). Here, we firstly found that isofraxidin could significantly shorten the escape latency and could ameliorate reductions in swimming times and crossing numbers within target quadrants in the Morris water maze test in a dose-dependent manner. Based on current data, we suggested that isofraxidin was able to improve hippocampal functions and enhance spatial learning and long-term memory in animals under scopolamine insults. Besides, the Y-maze task was also used in our study. Spontaneous alternation behavior detected in Y-maze is supported to minimize motivational or emotional actions, which was used to detect working and short-term memory in animal models(Bajo et al. 2015). Here, we found that isofraxidin administrations could significantly improve scopolamine-induced impairments of spontaneous alternation behavior in the Y-maze task, in a dose-dependent fashion.

The cholinergic system plays a critical role in learning function and memory formation(Lombardo and Maskos 2015; Xian et al. 2015; Chen et al. 2018). Usually, the cholinergic transmission is terminated mainly by Ach hydrolysis mediated by the AChE enzyme. Abnormalities in the cholinergic system, including a decrease of acetylcholine levels and an increase of AChE activity, always result in constant Ach deficiency, which contributes to memory impairments and cognition deficits in dementia(Lombardo and Maskos 2015; Xian et al. 2015). Thus, inhibition of AChE has been considered as a target for the treatment of AD(Lombardo and Maskos 2015; Xian et al. 2015). In line with the previous study that isofraxidin provided inhibitory actions on AChE activity *in vitro(*Ahmad et al. 2014), in the current study,

isofraxidin-mediated protective effects on AChE and ACh levels were also found in the hippocampus in scopolamine-treated mice. Thus, we suggested that isofraxidin-mediated modulation of cholinergic function could ameliorate learning and memory impairments in scopolamine-induced AD mice, and might contribute to AD prevention and treatment.

Oxidative stress is another well-characterized pathogenesis of neurodegenerative disorders(Lim et al. 2016; Um et al. 2018). The oxidative stress and damage are always observed in the CNS in patients suffering from AD, which suggests oxidative stress may be one of the earliest events in the onset and progression of AD(Butterfield 2018). Moreover, accumulating evidence also indicates that excessive oxidative stress in the brain is well associated with memory impairment in scopolamine-induced AD models(Butterfield 2018; Ponne et al. 2020). In our study, we firstly confirmed the anti-oxidative actions of isofraxidin in scopolamine-treated mouse hippocampus via determination of lipid peroxidation levels. Also, in line with the previous study that isofraxidin could enhance antioxidant enzyme activity(Huang et al. 2012), current data found that isofraxidin could reverse scopolamine-induced depletion of GSH levels and decreases of SOD and GSH-Px activity. The abnormal levels of the reactive oxygen species (ROS), the unbalanced activity of the antioxidant enzyme, and the increased activation of other oxidative components always induce cellular dysfunction and neuron apoptosis. Due to several limitations, ROS production was not detected in the current study (Kumar et al. 2012; Gan and Johnson 2014). Therefore, in our further study, the effects of isofraxidin on ROS generation should also be well studied. It is well reported that the NF-E2-related factor 2 (Nrf2) is one of the transcription factors modulate the endogenous antioxidant defensive systems and maintain the redox homeostasis to protect cells against oxidative stress (Kumar et al. 2012; Gan and Johnson 2014). Since that isofraxidin could confer anti-oxidative actions, it is necessary to explore the role of isofraxidin in Nrf2 pathways in our further study. In summary, the antioxidant activity by isofraxidin might prevent the hippocampus from scopolamine-induced oxidative damages and might alleviate behaviors impairments.

It is well reported that neuroinflammation is a kind of inevitable and essential pathological process in degenerative changes and cognitive impairment associated with AD(Xanthos and Sandkuhler 2014; Mietto et al. 2015). Accumulating evidence demonstrates that scopolamine treatment leads to neuroinflammatory responses by promoting proinflammatory mediators and cytokines expression in the hippocampus(Liu et al. 2017). Pro-inflammatory cytokines, like TNF-α, IL-6, and IL-1β, play essential roles in several events in the pathological cascade of AD(Selles et al. 2018). These cytokines could promote neuronal damages and induce more microglia activations as feedback. Besides, pro-inflammatory cytokines are also reported to implicate dysfunction of the cholinergic system via increasing AChE activity(Liu et al. 2017; Selles et al. 2018; Onasanwo et al. 2021), which in turn deteriorate neurodegenerative diseases. Hence, modulating cytokine functions is key to control inflammation-induced cognitive impairments. In the current study, the inhibitory effects of isofraxidin on pro-inflammatory cytokines production were well studied. In summary, we suggested that isofraxidin inhibitory actions on pro-inflammatory cytokines expression in the scopolamine-induced hippocampus might contribute to cognitive-improving actions in behaviors test.

CREB is one of the best understood phosphorylation-dependent cellular transcription factors. Normally, CREB binds to promoter regions of genes that are closely associated with neuronal differentiation, neurogenesis, synaptic plasticity, and cognitive activity in the CNS. The phosphorylation of CREB is necessary for memory storage and formation(Rosenblum et al. 2000; Wu et al. 2019). The abnormality of CREB signaling has been implicated in pathological conditions of AD(Rosenblum et al. 2000; Wu et al. 2019). In addition, BDNF is an essential member of the nerve growth factor family. BDNF and its receptor, TrkB, are broadly expressed in the developing and adult mammalian brain. The BDNF/TrkB-stimulated intracellular signaling plays a crucial role in neurotransmitter releases, synaptic plasticity, postsynaptic response, and cognitive processes(Um et al. 2018). Recent studies have indicated that altered levels of BDNF in patients and animals suffering from AD, and BDNF level has been used as a specific biomarker of AD(Rosenblum et al. 2000). Furthermore, several agents are reported to achieve their efficacy via promoting BDNF level further elicit their actions in animal models(Liu et al. 2017; Selles et al. 2018). It is well known that binding of BDNF to TrkB elicits various intracellular signaling pathways(Liu et al. 2017; Selles et al. 2018). ERK is a well-reported downstream signal transduction protein of BDNF and takes part in the development of memory formation and cognitive processes(Liu et al. 2017; Selles et al. 2018). Several agents beneficial for AD have been reported to up-regulate the level of phosphorylated ERK, indicating that ERK is well associated with the management of AD(Liu et al. 2017; Selles et al. 2018). Moreover, ERK inhibition results in deficits in long-lasting forms of synaptic plasticity and impairment of memory formation(Tancredi et al. 2000). Since BDNF, CREB, and ERK are involved in learning and memory processes, agents that provide effects on the activation of CREB and ERK and increase of BDNF level might have promising potential for developing novel anti-AD agents. In the current study, we further confirmed the role of isofraxidin in modulating BDNF, TrkB, CREB, and ERK in scopolamine-induced mice, suggesting that cognitive-improving actions of isofraxidin may be related to the mitigation of scopolamine-induced TrkB, CREB, and ERK inactivation and BDNF decline. Due to several limitations, we did not investigate the isofraxidin actions on synaptic plasticity in the current study. Since the BDNF-CREB-ERK pathways were well associated with synaptic plasticity, we suggest that it is expectable to explore the role of isofraxidin-mediated activation of BDNF-CREB-ERK in our further study.

Conclusions

The key findings of this study included (as illustrated in Fig. 8), 1. Isofraxidin supplementation could significantly improve scopolamine-induced behavioral impairments and cognition deficits in mice; 2. Isofraxidin could facilitate cholinergic activity, alleviate oxidative stress, and inhibit the inflammatory response in the hippocampus in a dose-dependent fashion; 3. Isofraxidin could up-regulate BDNF expression and promote phosphorylation of TrkB, ERK, and CREB in the hippocampus. In summary, isofraxidin conferred protective effects against scopolamine-induced memory and cognitive impairments in mice, which in part, were well associated with improvement of cholinergic activity, suppression of oxidative stress and inflammation response, and modulation of the BDNF-CREB-ERK pathway. These results indicated that isofraxidin might be a candidate drug for preventing and treating memory and cognitive dysfunctions.

Declarations

Author contributions

Designed the study: Bingliang Lian and Xiaoli Wu. Performed the experiments:Bingliang Lian, Jingwen Gu, Chen Zhang, Zhicong Zou and Meng Yu. Data collection and Statistical analyses: Bingliang Lian, Jingwen Gu, Chen Zhang, Zhicong Zou and Meng Yu. Wrote the main manuscript text: Bingliang Lian and Xiaoli Wu. Revised the main manuscript text: Fanghong Li and Allan Zijian Zhao. All authors reviewed and approved the manuscript.

Disclosure statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

Data availability statement

The data that support the findings of this study are available on requests from the corresponding authors.

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Figures

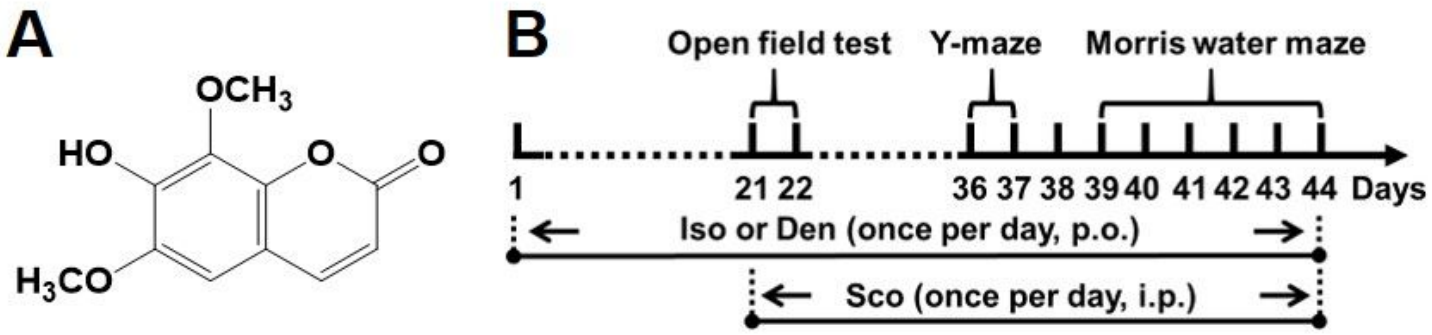

Figure 1

Chemical structure of isofraxidin and experimental schedule. (A) Chemical structure of isofraxidin; (B) The experimental schedule. Isofraxidin (Iso, 3, 10, and 30 mg/kg, once per day, p.o.) or donepezil (Don, 10

mg/kg, once per day, p.o.) was administered to mice for 44 days. Scopolamine insults (Sco, 1 mg/kg, once per day, i.p.) was given from the 21st to the 44th day.

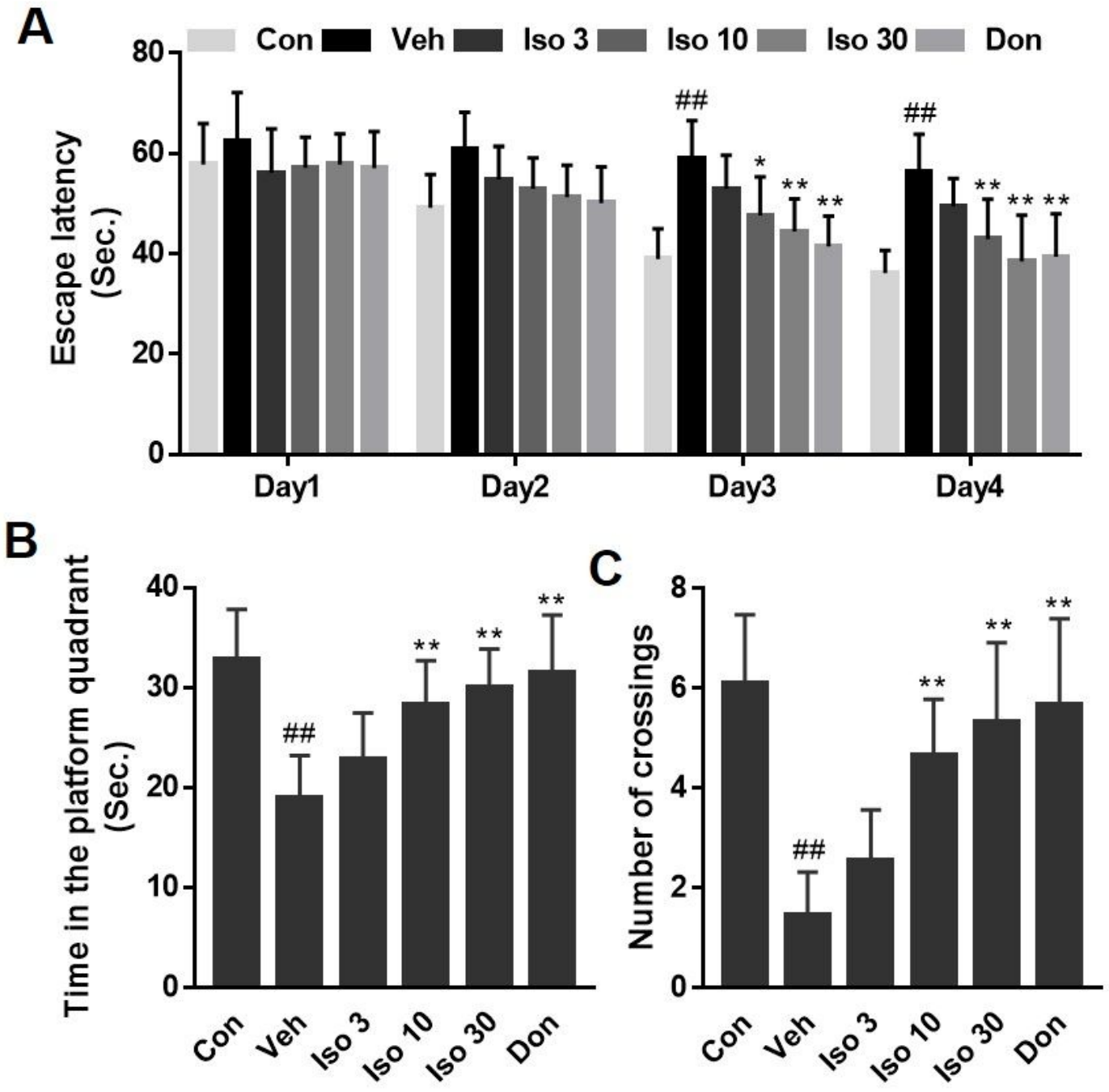

Figure 2

Effects of isofraxidin on scopolamine-induced cognitive deficits and memory impairments in the Morris water maze test. The Morris water maze test was performed from the 39th to the 44th day. (A) Latency to reach the escape platform; (B) Time spent in the platform quadrant. (C) The number of platform crossings. All data are presented as the mean ± SD (n = 9). Significance was analyzed by repeated-

measures one-way ANOVA (for A) and one-way ANOVA (for B and C) followed by Newman-Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

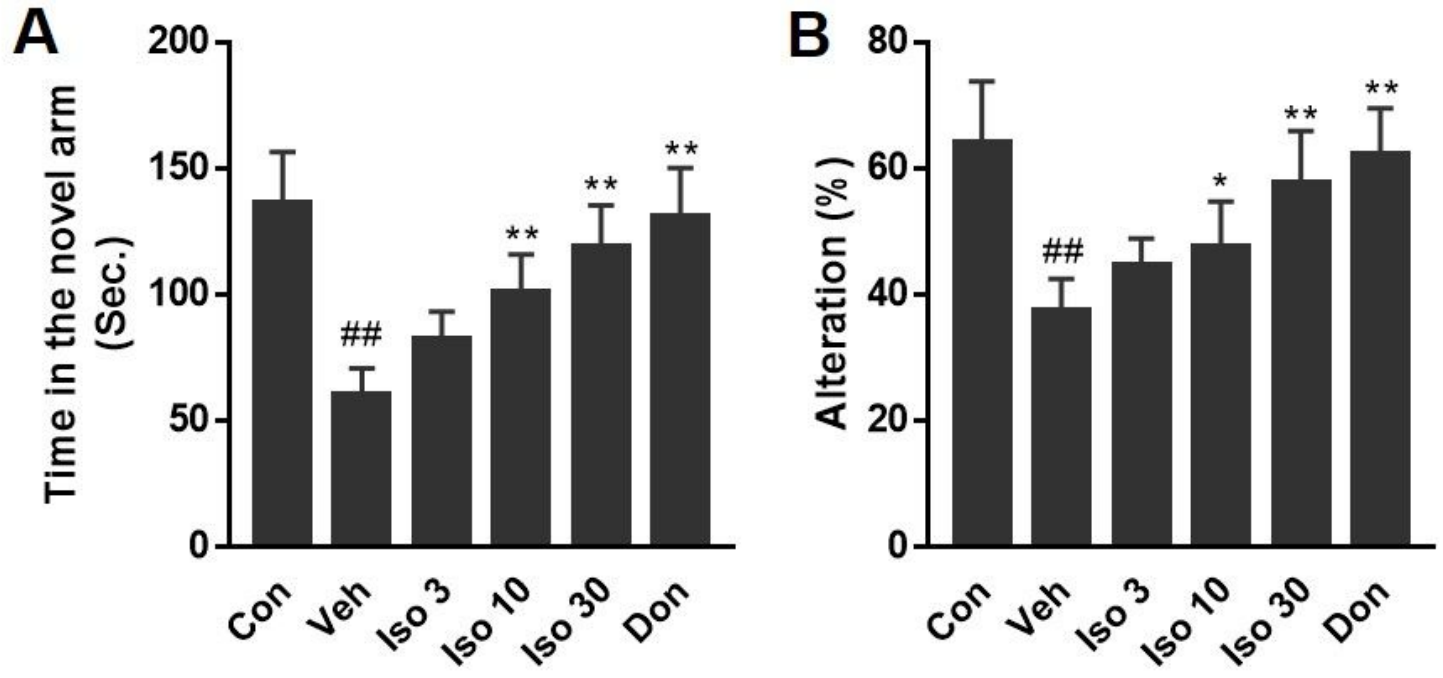

Figure 3

Effects of isofraxidin on scopolamine-induced cognitive deficits and memory impairments in the Y-maze test. The Y-maze test was conducted from the 36th to the 37th day. (A) Time spent in the novel arm; (B) Percentage of alterations. All data are presented as the mean ± SD (n = 9). Significance was analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

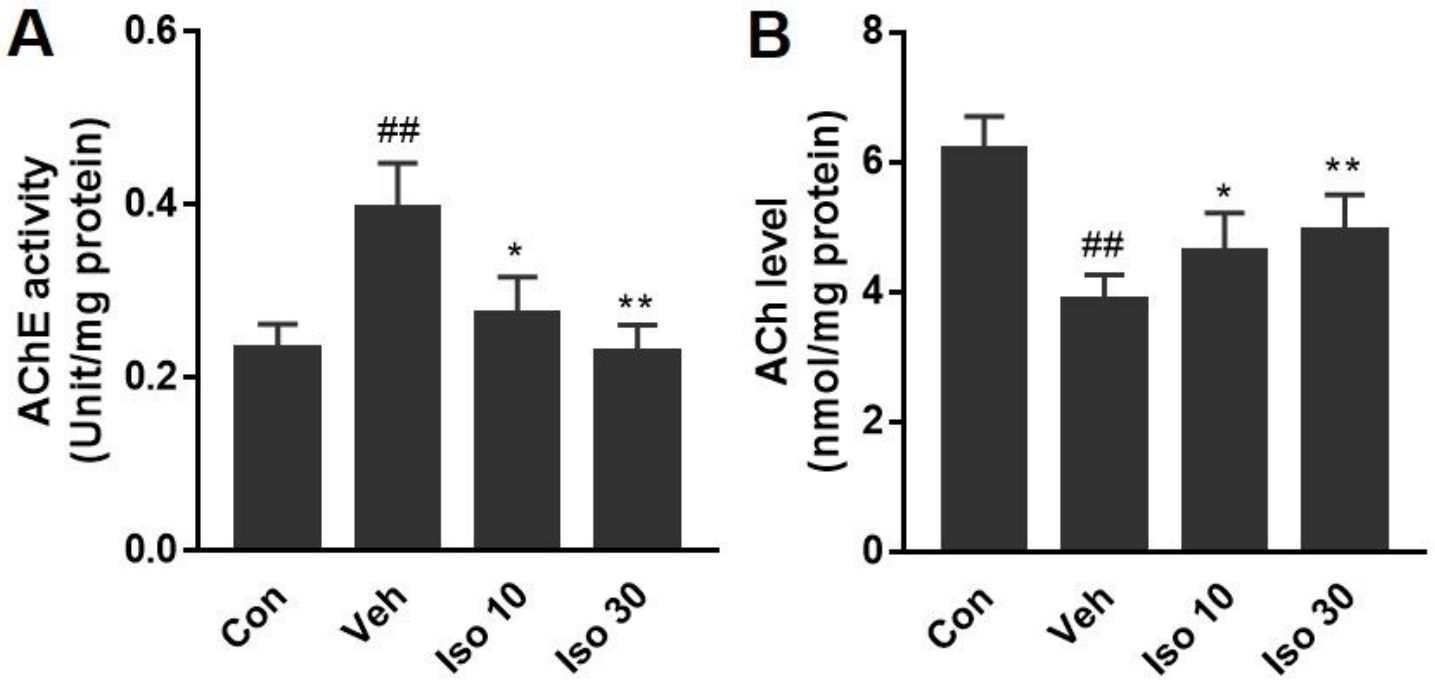

Figure 4

Effects of isofraxidin on acetylcholinesterase activity and acetylcholine level. On the 44th day, mice were sacrificed, and samples were collected for analysis. (A) The acetylcholinesterase (AChE) activity of the hippocampus; (B) The acetylcholine (Ach) level of the hippocampus samples. All data are presented as the mean ± SD (n = 9). Significance was analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

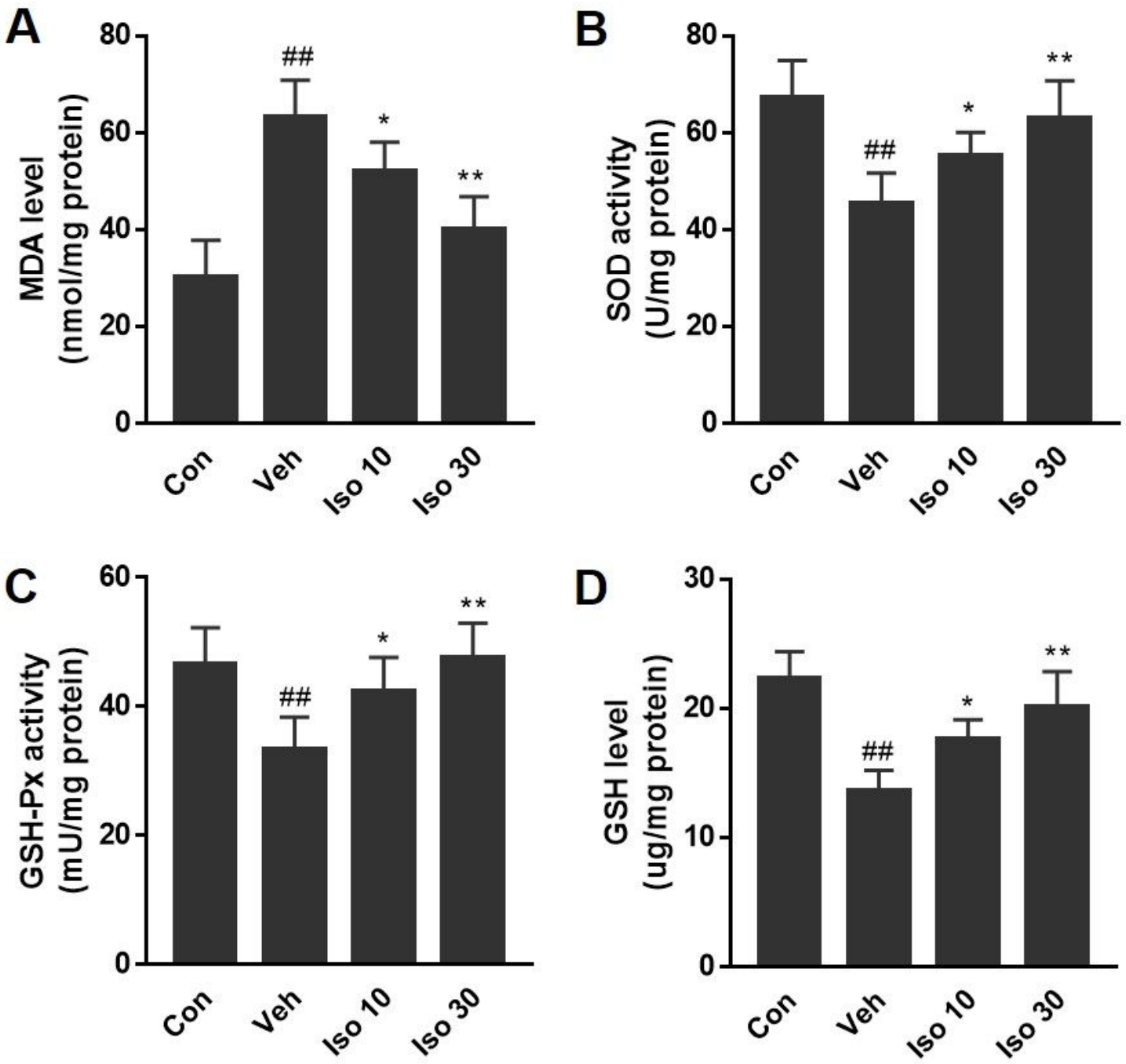

Figure 5

Effects of isofraxidin on oxidative stress in the hippocampus of scopolamine-induced mice. On the 44th day, mice were sacrificed, and samples were collected for analysis. (A) MDA level; (B) SOD activity; (C) GSH-Px activity; (D) GSH level. All data are presented as the mean ± SD (n = 9). Significance was analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

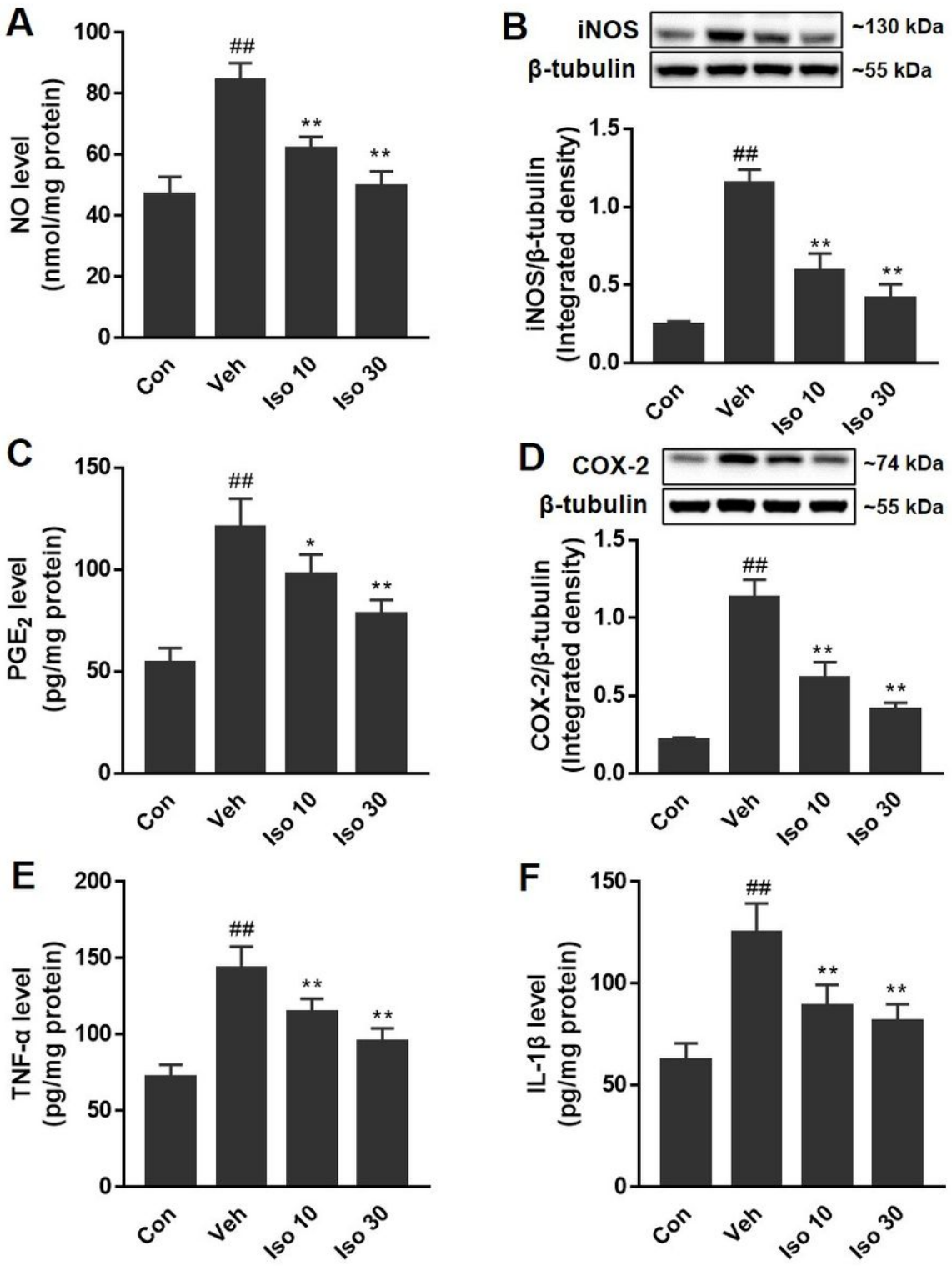

Figure 6

Effects of isofraxidin on inflammatory mediators and cytokines in the hippocampus of scopolamine-induced mice. On the 44th day, mice were sacrificed, and samples were collected for analysis. (A) NO level; (B) iNOS expression; (C) PGE_2 level; (D) COX-2 expression; (E)TNF-α; (F) IL-1β. All data are presented as the mean ± SD (n = 9). Significance was analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

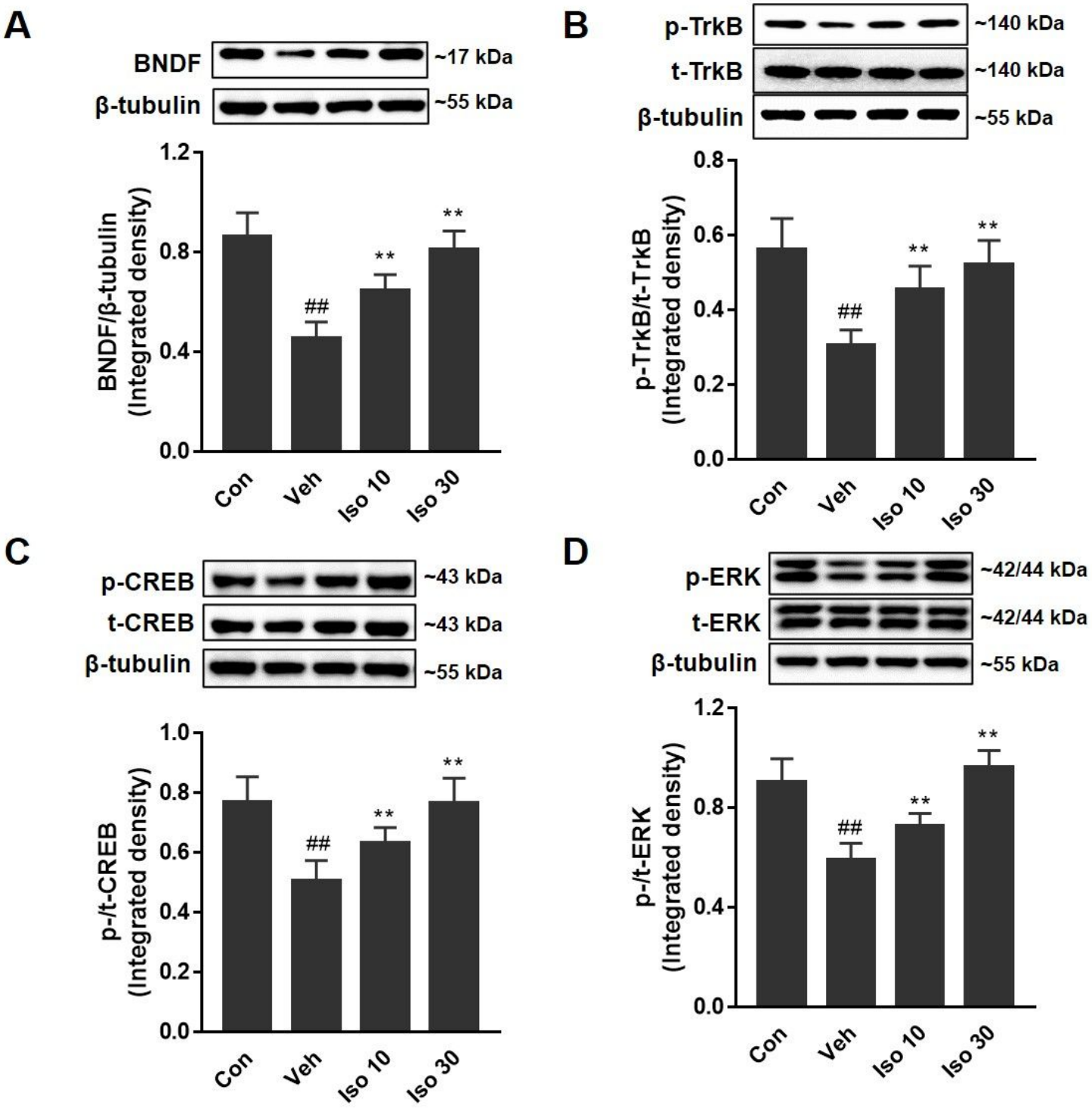

Figure 7

Effects of isofraxidin on the expression of BDNF and phosphorylation of TrkB, CREB, and ERK in the hippocampus of scopolamine-induced mice. On the 44th day, mice were sacrificed, and samples were collected for analysis. Western blotting and densitometric quantification of (A) BNDF, (B) phosphorylated TrkB, (C) phosphorylated CREB and total CREB, and (D) phosphorylated ERK and total ERK. All data are presented as the mean ±SD (n = 9). Significance was analyzed by one-way ANOVA followed by Newman-

Keuls *post hoc* test. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus vehicle group.

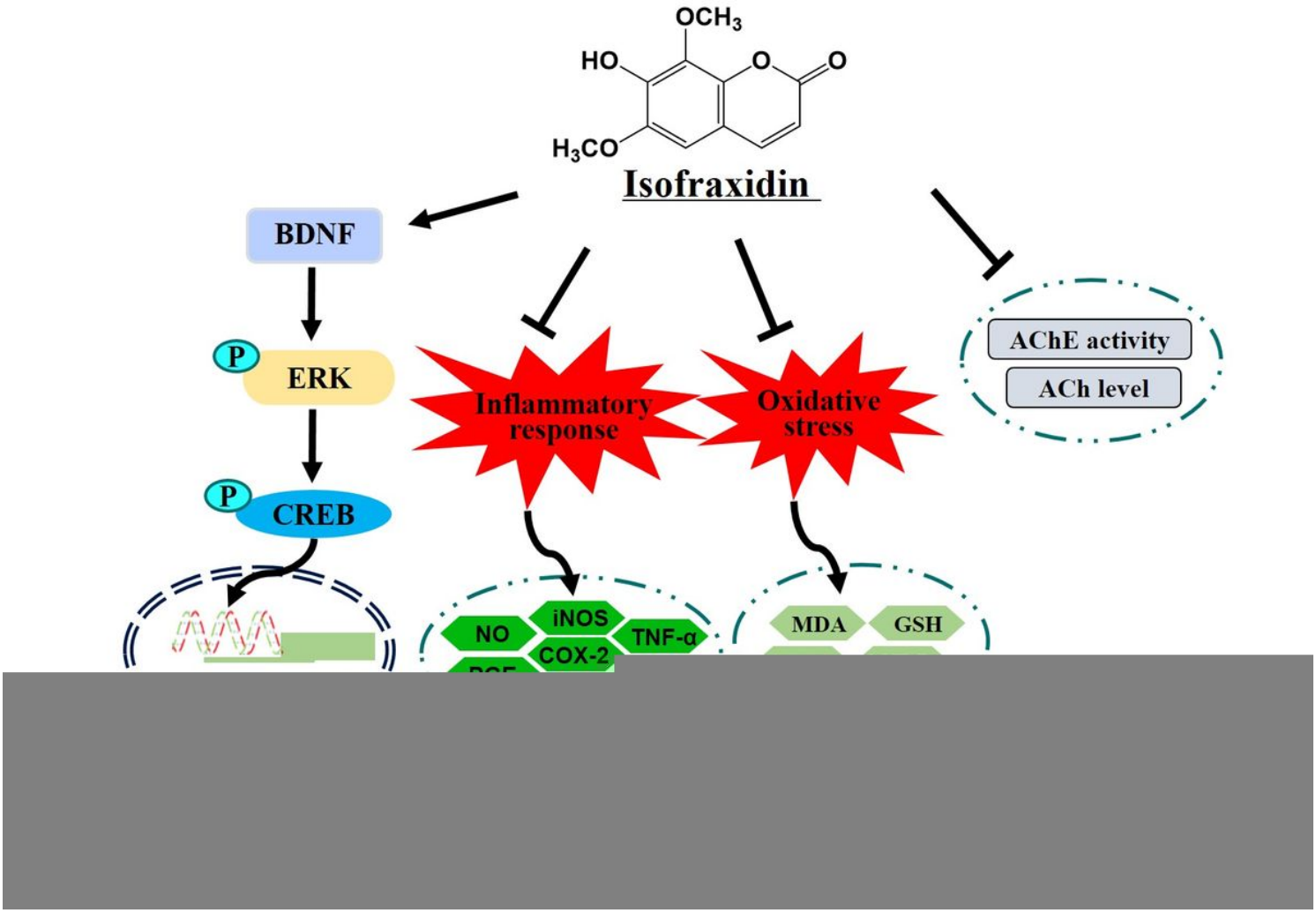

Figure 8

Schematic suggesting the potential mechanism for protective effects conferred by isofraxidin in scopolamine-induced mice.