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Original Research

The Protective Effects of Ecdysterone on Cognitive Impairment through Regulating Akt/GSK-3β/Nrf2 Signaling Pathway and Oxidative Stress in Cognitive Mice Model and Aβ-Induced Cell Neurotoxicity

Haihui Xing[1], Guohua Chen[2], Faisal Raza[3], Hajra Zafar[3], Lei Xing[4], Qiaoyun Li[1], Ran Li[5], Shanshan Xu[6,*], Dan Chen[7,*]

[1]Department of Neurology, Nanjing Gaochun Hospital of Traditional Chinese Medicine, 211300 Nanjing, Jiangsu, China
[2]Department of Surgical, Nanjing Gaochun Hospital of Traditional Chinese Medicine, 211300 Nanjing, Jiangsu, China
[3]Department of Pharmacy, School of Pharmacy, Shanghai Jiao Tong University, 200240 Shanghai, China
[4]Department of Geriatrics, Nanjing Gaochun Hospital of Traditional Chinese Medicine, 211300 Nanjing, Jiangsu, China
[5]Department of Pharmacy, School of Pharmacy, Jiangsu University, 212013 Zhenjiang, Jiangsu, China
[6]Department of Neurology, People's Hospital of Gaochun, 211300 Nanjing, Jiangsu, China
[7]Department of Rehabilitation Medicine, Xuancheng People's Hospital, 242000 Xuancheng, Anhui, China
*Correspondence: 791762276@qq.com (Shanshan Xu); caroline_0108@163.com (Dan Chen)

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Abstract

Background: Severe neurological condition like Alzheimer's disease (AD) has a significantly negative impact on families and society, wherein there is no proven cure. As one of the principal active constituents of *Achyranthes bidentata* Blume, ecdysterone (ECR) has demonstrated antioxidant and cognitive dysfunction improvement effects. Nonetheless, the mechanism underlying the improvement of cognitive dysfunction by ECR remains unclear. This study sought to ascertain whether ECR may allebviate cognitive impairment by reducing oxidative stress via activation of the nuclear factor erythroid-2-related factor-2 (Nrf2) antioxidant system through Akt/GSK3β pathway. **Methods**: In terms of the experimental procedure, we determined the neuroprotective benefits of ECR *in vivo* via a cognitive impairment model of senescence-accelerated mouse prone 8 (SAMP8), we performed procedures such as behavioral testing, biochemical assaying, Nissl and TUNEL stainings, as well as flow cytometry, immunohistochemistry and western blotting. Furthermore, we investigated the underlying mechanistic action of ECR by activating PC12 cells with β-amyloid peptide fragment 25-35 (Aβ25-35). **Results**: *In vivo* studies showed that ECR effectively improved cognitive impairment in SAMP8 via enhancement of learning and memory capabilities, but decreased oxidative stress, apoptosis and neuronal damage in the hippocampus. During the *in vitro* study, we observed that ECR dose-dependently reduced the oxidative stress and apoptosis that were induced in PC12 cells by Aβ25-35. Additionally, the use of Akt inhibitors further established the potential of ECR to control Nrf2 through activation of the Akt/GSK3β pathway and protect the PC12 cells from Aβ25-35 induced damage. **Conclusions**: These findings offer proof that ECR reduces cognitive impairment by triggering the Nrf2 antioxidant system via the Akt/GSK3β pathway and offer fresh information on ECR's potential as a promising therapeutic development candidate for AD.

Keywords: Alzheimer's disease; ecdysterone; oxidative stress; Nrf2; Akt/GSK3β

1. Introduction

One of the most predominant disorders of the central nervous system that poses significant problems within the realm of global public health is Alzheimer's disease (AD), which is a neuro-degenerative condition that is associated with cognitive and memory loss [1]. Amyloid plaques and intracellular neurofibrillary tangles (NFT), two significant neuropathological signs, are its defining characteristics [2,3]. As the number of AD patients rises, their families experience considerable misery, and society as a whole is heavily burdened socioeconomically [4]. Currently, the fight against AD is neurology's most pressing unmet medical need. For a long time, there has been no specific cure for AD, only a comprehensive approach to controlling the progression of the disease [5]. Therefore, it is critical in the realms of public health to develop effective treatment interventions for AD.

The pathophysiology of AD is heavily influenced by oxidative stress [6]. It contributes to Aβ deposition, tau hyperphosphorylation, and subsequent synapses and neuronal death in the onset of AD [7]. Illustratively, elevated levels of Aβ1-40 and Aβ1-42 have been demonstrated to connect to increased amounts of oxidative byproducts of lipids, nucleic acids and proteins in the cortex and hippocampus of AD [8]. Moreover, a decrease in superoxide dismutase (SOD)1 (cytoplasmic isoform) was decreased or

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a deficiency in SOD2 (mitochondrial isoform) resulted in an increase in tau phosphorylation in Tg2576 AD transgenic mice [9]. Oxidative stress has been associated with AD, which suggests that the former is significantly involved in the pathological process of the latter. Thus, the reduction of oxidative stress can be very crucial in the treatment of AD.

The cellular antioxidative response is thought to be mainly regulated by the nuclear factor erythroid-2-related factor-2 (Nrf2) [10]. Normally, Nrf2 is localized in cytoplasm, but it is translocated to the nucleus after exposure to oxidatively stress conditions, where it activates genes that protect against oxidation [11]. Due to Nrf2's critical role in neuroprotection in AD, its deletion or mutation worsens memory loss, cognitive decline, and Aβ pathology [12,13]. It has been observed that when the *Nrf2* gene was knocked out in amyloid precursor protein (APP) transgenic mice of AD animal model, deficits of cognitive, memory and spatial learning of model mice were significantly aggravated [14]. In comparison with brains in healthy individuals, it is well known that Nrf2 signaling was diminished in the brains of AD patients, with a particular reduction in Nrf2 expression in the nuclear compartment of neurons in the hippocampus [15]. As a result of the above assertion, Nrf2 is crucial in AD treatment. Besides, Nrf2 is the target of the Akt/GSK3β pathway, one of the numerous upstream signaling pathways [16]. Phosphorylation of Akt activates GSK3β, promotes the transfer of Nrf2 from Keap1-binding sites to the nucleus, and then inhibits oxidative stress by transactivating downstream target genes via AREs [17]. Several studies have shown that the Akt signaling pathway plays a key role in AD. For example, Lee *et al.* [18] have shown that fucoxanthin exerts resistance to amyloid-beta peptide-induced oxidative damage through the Akt/GSK-3β signaling pathway. Xiong *et al.* [19] showed that BMSCS-exosomes containing GDF-15 alleviated the SH-SY5Y cell damage model of AD through Akt/GSK-3β. Together, the Akt/GSK3β signaling pathway may be crucial in AD treatment via Nrf2.

In traditional Chinese medicine, the roots of the *Amaranthaceae* plant *Achyranthes bidentata* Blume are frequently used to treat dementia [20]. Ecdysterone (ECR) is one of the main active ingredients of *Achyranthes bidentata Blume* and its discovery has increased medicinal value while its antioxidant activity has been reported [21]. Additionally, earlier research has demonstrated that ECR can enhance rat C-fos expression, alleviate cognitive impairment brought on by oral administration of the β-amyloid peptide fragment 25-35 (Aβ25-35), and facilitate learning and memory. The gene, which measures neuronal activity, is directly linked to memory and learning in the cerebral cortex and hippocampus [22]. Furthermore, the complimentary pathways linked with c-Jun N-terminal kinase and Akt have also been demonstrated by Xu *et al.* [23] to be the mechanism by which ECR shields SH-SY5Y cells from β-amyloid-induced apoptosis. However, it is not clear whether ECR regulates Nrf2 in an Akt/GSK3β-dependent manner to inhibit oxidative stress and thus improve cognitive impairment.

Based on available literature, it was postulated that ECR may regulate Nrf2 in an Akt/GSK3β-dependent manner to inhibit oxidative stress and thus improve cognitive impairment. Therefore, we sought to explore the neuroprotective activity of ECR using the AD model of Aβ25-35 treated PC12 cells and senescence-accelerated mouse prone 8 (SAMP8). Besides, elucidation of the potential mechanism of ECR in AD treatment was carried out by investigating its effect on the Akt/GSK3β and Nrf2 antioxidant systems.

2. Materials and Methods

2.1 Chemicals and Antibodies

Abcam (Cambridge, UK) provided antibodies for Aβ-1 (cat. no. ab201060), BCL-2 (cat. no. ab182858), Bax (cat. no. ab32503), HO1 (cat. no. ab1346) and Nrf2 (cat. no. ab62352), while cell signaling tech., (Danvers, MA, USA) supplied antibodies for p-tau (cat. no. 12885), Akt (cat. no. 9272), P-Akt (cat. no. 4060), GSK3β (cat. no. 12456), P-GSK3β (cat. no. 5558), LaminB1 (cat. no. 17416), caspase-3 (cat. no. 9662S), cleave caspase-3 (cat. no. 9664S), GAPDH (cat. no. 5174), and goat anti-rabbit (cat. no. 14708) and mouse (cat. no. 14709) IgG (H+L) HRP. Affinity (Melbourne, FL, USA) provided ECL reagent (cat. no. KF8003), while Sigma-Aldrich (St. Louis, MO, USA) supplied Dulbecco's modified-Eagle medium (DMEM) and fetal bovine serum (FBS). Also, we obtained trypsin, multicolor protein marker, sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) kit and 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) dye from Solarbio (Beijing, China). Millipore (Millipore, Bedford, MA, USA) supplied polyvinylidene fluoride membranes (PVDF). The BCA protein concentration assay (Enhanced) (cat. no. P0010) and One-step TUNEL cell apoptosis detection kit (cat. no. C1089) were provided by Beyotime (Shanghai, China). Malondialdehyde (MDA) (cat. no. ml094962), Superoxide dismutase (SOD) (cat. no. ml092620), and reduced glutathione (GSH) (cat. no. ml092952) content assay kits were supplied by Elisa (Shanghai, China). Macklin Biochemical Co., Ltd. (Shanghai, China) provided ECR (purity 98%) (cat. no. H811108) and Donepezil (cat. no. D849374).

2.2 Experimental Animals

Changzhou Cavins Laboratory Animal Co., Ltd. (Changzhou, China) provided the male mice, namely senescence accelerated-resistant mouse (SAMR1) and Senescence-accelerated mouse prone 8 (SAMP8), which were without any specific pathogen, 4 months old and weight 30–35 g. All the animals were fed in cages in the same quiet environment with a light (12 L)/dark (12

D) cycle, respectively fed and drank sterilized feed and water. The experiment was carried out after 7 days of adaptation. The Jiangsu University (UJS IACUC) institutional committee for the care and use of laboratory animals reviewed and approved (approval number: UJS-IACUC-2022031401) for studies involving animals.

2.3 Animal Grouping and Treatment

One week later, 48 qualified animals were selected, and 40 SAMP8 mice except for 8 SAMR1 mice in the control group were randomly divided into 5 groups. All the animals were divided into six groups: SAMR1 blank group (Control), SAMP8 Model group (Model), SAMP8 model + ecdysterone Low dose group (Low), SAMP8 model + ecdysterone Medium dose group (Medium), SAMP8 model + ecdysterone High dose group (High), SAMP8 model + Donepezil group (Positive). Mice in the administration group were given ECR intragastric administration (high, medium and low doses were 5, 10, and 20 mg/kg/day, respectively) [24]. The control group and model group were intragastrically given 0.9% sodium chloride solution of the same volume, and the positive group was intragastrically given Donepezil (1 mg/kg/day). The drug treatment group and the control group were fed the same way in different cages. And continued administration for 4 weeks. Animals were sacrificed after the treatment, and tissues were collected and kept in a –80 °C freezer until further analysis.

2.4 Cell Culture and Treatment

One of the most popular cell lines for neuroscience research is PC12, which is employed in investigations on synaptogenesis, neurotoxicity, neuroprotection, neurosecretion, and neuroinflammation [25]. STR profiling was used to confirm the PC12 Cell Line, and mycoplasma testing came out negative. Every cell was cultivated at 37 °C and 5% CO_2 in a humidified incubator. In order to cultivate PC12 cells, DMEM with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (C0222, Beyotime) was used. Later on, we categorized the cells into five groups, viz., control, model, low-dose ECR (25 µM), medium-dose ECR (50 µM) and high-dose ECR (100 µM). Only normal media of equal volume was used to cultivate the cells in the control category. The model group was treated with Aβ25-35 in oligomer form at 25 µM (A4559, Sigma) for 24 h to establish an *in vitro* AD model. Likewise, we employed Aβ25-35 (25 µM) to treat the cells in low, medium and high dose groups in normal medium for 24 h, before replacement with medium containing ECR (25, 50 and 100 µM) for another 24 h.

2.5 Testing of Mice Behavior

2.5.1 Navigation in Morris Water Maze (MWM)

In brief, we placed the platform in the middle of the fourth quadrant, while an automatic camera system with a computer connection was put in place above the pool to monitor and record the swimming activities of the mice. The mice's swimming distance and travel time to the platform (to avoid the incubation period) were automatically estimated. Mice were thrown into the water with their backs to the pool wall from one of the four starting places. We looked at the movement patterns within 90 s before timing the duration within which the mice found the platform. The experimenter took the mice to the platform, where they rested for 15 s before the next training when they could not find the platform inside 90 s (90 s was recorded as the escape incubation period). We trained the mice 4 times a day at a fixed time period. Within 4 times of training, the mice were put into water from 4 different quadrants, while the interval of each training was 1 min. After three days of continuous training, the formal experiment began. Eventually, we recorded the time taken by the mice from the time they were placed into the pool to the time they found the platform. Once a day for 5 consecutive days, we recorded the time to be 90 s when the time for the mice to find the platform exceeded 90 s.

2.5.2 Morris Water Maze Space Exploration Experiment

After the navigation experiment, the mice in each group rested for one day, and then tested their memory ability through the space exploration experiment. Mice were added to the pool from the third quadrant after the platform in the fourth quadrant was removed. Each mouse group was observed and counted as they passed the platform in the fourth quadrant over the course of 90 s.

2.6 Nissl Staining

Paraffin sections of mouse hippocampal tissue were dewaxed and immersed in water, dyed with 1% toluidine blue for 10 min, and rinsed with distilled water. The colour is then separated in 70% alcohol for seconds to minutes. Then in anhydrous ethanol dehydration, xylene is transparent. Finally, seal it with a neutral glue and observe the cornu ammonis 1 (CA1) and CA3 regions of the hippocampus under a microscope.

2.7 Immumohistochemical Staining

After administration, hippocampal tissue was taken from mice and placed in 4% (w/v) paraformaldehyde (PFA) solution overnight. Before cutting the tissue into slices (4 µm), we embedded them in paraffin. Dewaxing of paraffin sections to water was carried out, before antigen repairing, blocking and sealing as well as overnight incubation at 4 °C with primary antibodies of Aβ and P-Tau and afterwards with secondary antibodies. Later, we visualized the tissue with 3, 3′-diamino-benzidine tetrahydrochloride before counterstaining with hematoxylin, dehydration, mounting and imaging using a high-power microscope. Image J software (V1.8.0; LOCI, University of Wisconsin, Madison, WI, USA) was used for quantitative analysis of staining results.

2.8 Quantification of Reactive Oxygen Species (ROS) Levels in Tissues and Cells

Measurement of ROS levels in tissues: At the end of the last administration, the hippocampus tissues of mice were extracted and cut into pieces, and then completely soaked in 2 mL digestive fluid (DMEM containing 1 mg/mL collagenase IV and 1 mg/mL DNA enzyme I). The above mixture was then placed in a 37 °C water bath for digestion. Ice was added after 45 min to stop digestion. The digested brain tissue solution was run through a 40 µm cell filter in order to exclude cell masses and tissue masses that had not been adequately digested. To obtain a single-cell suspension, the filtrate was centrifuged at 1000 rpm for 5 min while the cell precipitation was resuspended in PBS. Wash with PBS twice, centrifuge at 1000 r/min for 5 min, remove the supernatant, add an appropriate volume of diluted DCFH-DA working liquid, and incubate at 37 °C for 30 min in the dark. Following incubation, the cells underwent two PBS washes in order to eliminate any remaining DCFH-DA from the cells. In 500 µL of PBS, the cells were suspended. The ROS positive rate was found using flow cytometry.

Measurement of ROS levels in cells: PC12 cells were placed in a 6-well plate, 3.5×10^6 cells/well, and adherent cultured for 24 h. 24 h after the preparation of the model *in vitro*, the medium containing different concentrations of ecdysterone was replaced. Following a 24-hour period, the original supernatant was disposed of, the cells in the 6-well plate underwent two PBS washes, the diluted DCFH-DA probe was applied, and the mixture was incubated for 15–30 min in a dark environment. After incubation, the supernatant was discarded and washed twice with PBS to remove the DCFH-DA probe that did not enter the cell. Add 1 mL of 0.02% pancreatic enzyme without EDTA, and when the cells become round, add PBS to terminate digestion, and gently blow the cells with a pipette to suspend the cells. Collect in EP tube, centrifuge at 3500 rpm for 5 min, and discard supernatant. After adding 1 mL of pre-cooled PBS at 4 °C to the fully suspended cells, they were centrifuged for 5 min at 3500 rpm, and the supernatant was disposed of. In 500 µL of PBS, the cells were suspended. Using flow cytometry, the ROS positivity rate was discovered.

2.9 Determination of Malondialdehyde (MDA), Superoxide Dismutase (SOD) and Reduced Glutathione (GSH) Levels

The isolated hippocampal tissue was homogenized in cold phosphate buffer (pH 7.4) and centrifuged at 4 °C at 10,000 rpm for 15 min. The centrifuged supernatant (serum, cell culture supernatant) was collected and 100 µL supernatant was added to the plate. The levels of MDA, SOD, and GSH in supernatant, serum, and cell culture supernatant were measured using specific kits according to the manufacturer's instructions.

2.10 Western Blotting

Extraction of total protein from hippocampus and determination of protein content were carried out respectively with RIPA lysis and BCA protein assay kit. Later on, we performed electrophoresis of protein (40 µg) for 1 h on SDS-PAGE (10%) to PVDF membrane with 120 V of transmembrane step. After being blocked with 5% BSA, membranes were incubated with the primary antibodies (BCL-2, Bax, HO-1, P-Akt, Akt, GSK3β, P-GSK3β, LaminB1, Cleave-caspase 3, caspase 3 and Nrf2) for a whole night at 4 °C. Before using goat anti-mouse antibody or goat anti-rabbit antibody that had been HRP-labeled for 1 h at room temperature, the membrane was subjected to three Tris Buffered Saline with Tween (TBST) washes. After three washes of the membrane with TBST, we quantified peroxidase-labelled protein bands with an ECL kit before assessment of protein intensity with Image J.

2.11 Immunofluorescence

Tissue or cell samples that have been fixed in PFA (4%) were subjected to drying, paraffin embedding, slicing, dewaxing, hydrating, antigen extraction, blocking, and all-night incubation at 4 °C with primary antibody. Afterwards, sections were subjected to three PBS washes before the FITC conjugate secondary antibody was used for their incubation for 1 h at 37 °C. Later on, an anti-fade mounting medium with 4, 6-diamidino-2-phenyl-indole (DAPI) was used to mount the slices after washing. Ultimately, we evaluated the sections with a fluorescent microscope.

2.12 TUNEL Staining

Following the manufacturer's instructions, TUNEL labeling was used to identify neuronal cell death in the hippocampal regions of mice. Under a fluorescent microscope, the sections were taken in pictures. To count the cells that were TUNEL positive, Image J was employed. Data was expressed as a ratio of the number of TUNEL positive cells to the square millimeter.

2.13 Cell Viability Assay

Assessment of the effects of various dosage forms on cell viability was accomplished with the MTT assay. Growing of PC12 cells was carried out in 96-well plates for 24 h with 8000 cells/well. Twenty-four (24) h after an *in vitro* cell model preparation, we exposed the cells to various amounts of drug-containing serum. Following a 24 h treatment period, we applied MTT (5 mg/mL, 20 µL) to each well, before the removal of supernatant after 4 h. Later on, we added DMSO (150 µL) to each well, while complete dissolution of dirty crystals was accomplished with 10 min of oscillations at low-speed. At a wavelength of 570 nm, we used an enzymatic-labeled meter to determine the absorbance OD values.

2.14 Flow Cytometry for Apoptosis Analysis

According to the manufacturer's instructions, we detected cell apoptosis with Annexin V-FITC/propyl iodide (PI) apoptotic kit. To put it simply, we inoculated PC12 cells into 6-well plates with 3.5×10^6 cells/well density, before culturing for 24 h. After 24 h of preparation, the cultured medium containing different concentrations of ECR was changed and treated for 24 h. Afterwards, we used a binding buffer to digest, collect, centrifuge and resuspend the cells. At ambient temperature without any light exposure, we incubated the mixture after the addition of Annexin V-FITC (5 µL) and PI (5 µL). Later on, we examined the apoptotic cells using flow cytometry and the program FlowJo (V7.1.0; TreeStar, Ashland, OR, USA).

2.15 Statistical Analysis

To do the statistical analysis, GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) was used. We expressed the data with mean $\pm$ SD. One-way or two-way analyses of variance (ANOVA) were used to assess all of the data. Statistically, the accepted significant level was $p < 0.05$, while acceptance at $p < 0.01$ or $p < 0.001$ was regarded as being very significant.

3. Results

3.1 ECR Improves Cognitive Deficits in SAMP8 Mice

The aging process of SAMP8 mice is accompanied by complex physiological changes related to cognitive dysfunction, such as brain Aβ deposition, increased oxidative stress, Tau hyperphosphorylation and neuroinflammation, which is currently recognized as a natural senescence dementia model [26]. In this regard, we assessed the potential of ECR to improve cognitive impairment *in vivo* using SAMP8 and SAMR1 as the respective mice models and controls. Usually, MWM which includes experiments such as navigation and exploration of space exploration by laboratory animals, is a well-known approach for testing spatial learning and memory in trials of these animals [27]. We first employed MWM to assess the learning and memory capacity of mice to determine the impact of ECR on cognitive deficits in SAMP8 mice. According to Fig. 1A, the escape latency of the model group was much higher during the first 5 days compared to that of the control group, and the learning impairment of SAMP8 mice was improved after ECR or positive drug administration, and ECR was dose-dependent. On the last day of the space exploration experiment, mice in the ECR and positive groups increased the number of crossings in the platform region (Fig. 1B) and the time spent in the target quadrant region (Fig. 1C) compared to the model group. Additionally, we tested SAMR1 mice using MWM but found no significant effect on their behavior due to ECR treatment (**Supplementary Fig. 1**). Nissl staining was performed to evaluate histopathological abnormalities in the hippocampus, which are frequently linked to the course of the disease. As shown in Fig. 2A–C, SMAP8 mice displayed considerably fewer intact neurons in hippocampal regions (CA1 and CA3) compared with SAMR1 mice. Nonetheless, we observed a reversal of the above alteration after ECR and positive drug treatment, wherein amid ECR effect was in dose dependent fashion. In addition, immunohistochemical staining showed that Aβ and P-tau expressions in the hippocampus of model mice were increased markedly compared to control mice, but we observed a reversal of this increase after ECR and positive drug treatments (Fig. 2D–F). These data indicate that ECR effectively alleviated memory loss and learning disabilities in SAMP8 mice.

3.2 Impacts of ECR on Oxidative Stress in SAMP8 Mice

Scientists have shown that oxidative stress has a significant influence on the development of AD [7]. Based on this assertion, we evaluated oxidative stress-linked biomarkers to ascertain the involvement of this process in the ability of ECR to ameliorate cognitive deficits in SAMP8 mice. As shown in Fig. 3A,B, ROS levels considerably increased in the hippocampus of SAMP8 mice compared to the control group but decreased significantly after treatment with ECR or Donepezil, and ECR showed a dose-dependent manner. Important indexes that reflect oxidative system imbalance are GSH, MDA and SOD. As can be seen from Fig. 3C–E, SOD (Fig. 3D) and GSH (Fig. 3E) activities in the hippocampus of the model group were significantly decreased, while MDA (Fig. 3C) content was increased. These changes were significantly reversed by ECR or Donepezil, with a stronger reversal effect observed at higher doses of ECR. Similarly, the detection of MDA, SOD and GSH levels in the serum of mice was consistent with that in the hippocampus (Fig. 3F–H). Thus, inhibition of oxidative stress by ECR may facilitate its ameliorating effect on cognitive deficits in SAMP8.

3.3 ECR Inhibits Neuron Apoptosis in SAMP8 Mice

Much evidence suggests that peroxidation of lipids and proteins is the consequence of oxidative system imbalance, which ultimately culminates in apoptosis in cells [28]. To investigate whether inhibition of oxidative stress by ECR may further result in reduced cell apoptosis, we evaluated cell apoptosis and expression levels of apoptotic-linked protein in the hippocampus of the mice. To achieve this, we employed TUNEL staining to observe the ECR effect on the apoptosis of neurons in the hippocampus. In terms of results, we discovered a substantially increased number of apoptostic-positive cells in model mice compared to control, with the cells displaying distinctive morphological features of cellular apoptosis. Meanwhile, we observed a significantly decreased number of TUNEL positive cells in the hippocampus of mice that received ECR compared to model mice (Fig. 4A,B). Following that, we ascertained the alterations in proteins that have been linked

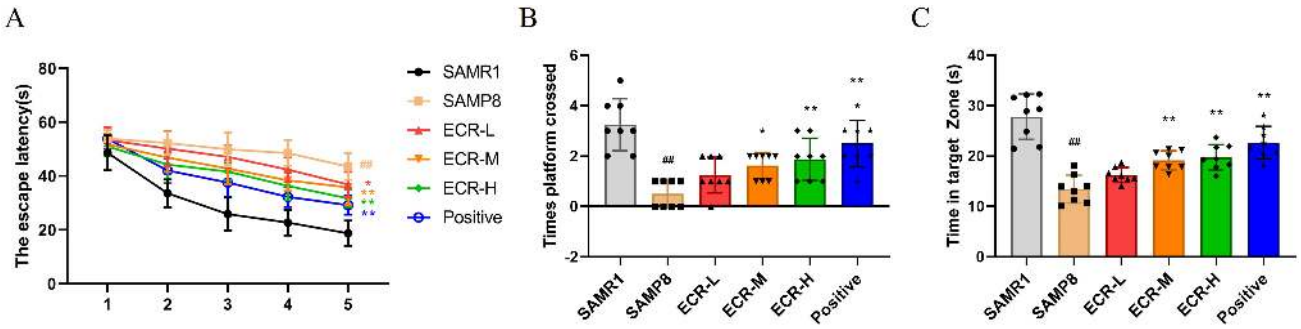


Fig. 1. ECR improves cognitively deficient behavior in SAMP8 mice. Effects of ECR treatment on (A) escape latency, (B) number of times crossed the target platform position and (C) time spent in the target quadrant in Morris water maze (MWM). (A) Data were analyzed using two-way ANOVA and a Bonferroni test or (B,C) using a one-way ANOVA and Tukey's post hoc test and presented as the mean ± standard deviation (SD), n = 8 in each group. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice. ECR, ecdysterone; SAMP8, senescence-accelerated mouse prone 8; ANOVA, analyses of variance.

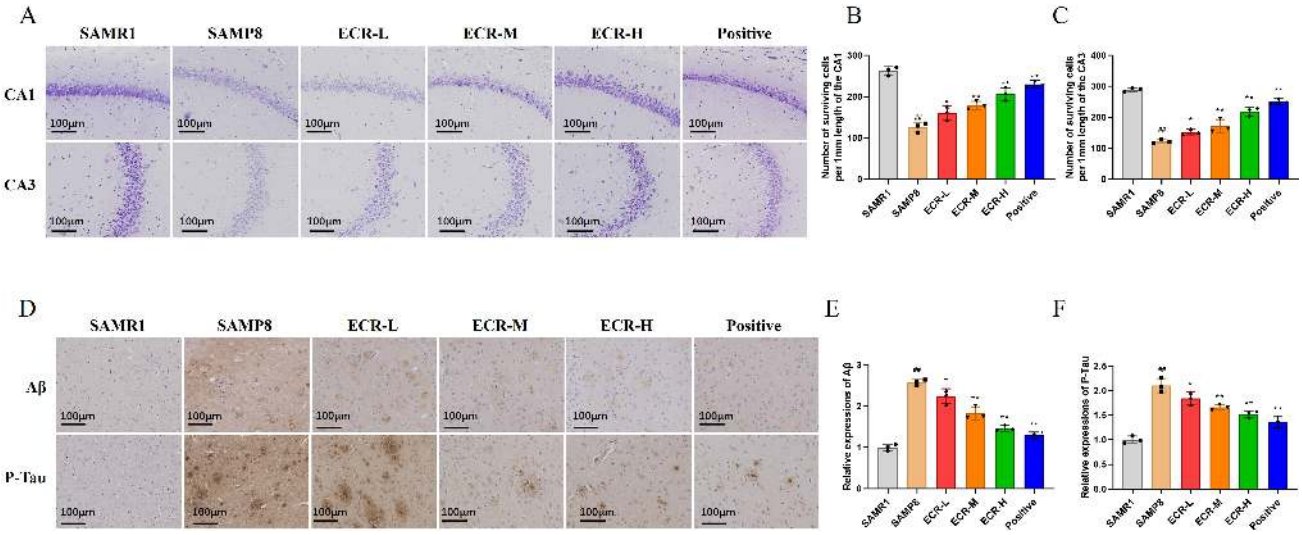


Fig. 2. ECR improves cognitive deficits in SAMP8 mice. (A) Illustration of images of Nissl staining of hippocampal regions (CA1 and CA3). Surviving cells per 1 mm of (B) CA1 and (C) CA3 were analyzed quantitatively. In each group, n = 3, while the scale bar = 100 μm. (D) By using immunohistochemical staining, Aβ and P-Tau expressions in the hippocampus were discovered. (E,F) Quantitative analysis of immunohistochemical staining. In each group, n = 3, while the scale bar = 100 μm. The data were presented as the mean ± SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice.

to apoptosis with the western blotting technique. Regarding the findings, we saw that model mice expressed less Bcl-2 than control mice did, while the former group expressed more Bax and cleaved caspase-3 (Fig. 4D,E) . Nevertheless, ECR treatment could significantly reverse these changes, wherein the reversal was more obvious with an increase in ECR dose (Fig. 4C,F). These data suggest that, in the hippocampus of SAMP8 mice, ECR has a protective impact against cell apoptosis, and that this protective effect increased with increasing ECR concentration within a specific range.

3.4 ECR Improves Cognitive Deficits in SAMP8 Mice by Activating Akt/GSK3β to Regulate Nrf2

Immunofluorescent staining and western blotting techniques were utilized to detect the expression of proteins linked to the Akt/GSK3β signaling pathway and Nrf2 to confirm that the potentiality of ECR to improve cognitive deficits in SAMP8 involves inhibition of oxidative stress and neuronal apoptosis. The immunofluorescence results (Fig. 5A–C) imply that Akt and P-GSK3β expression levels in model mice reduced considerably compared to control mice, but ECR treatment could partially reverse this effect. Additionally, there was no discernible change in the level of Nrf2 expression between the model group and the control

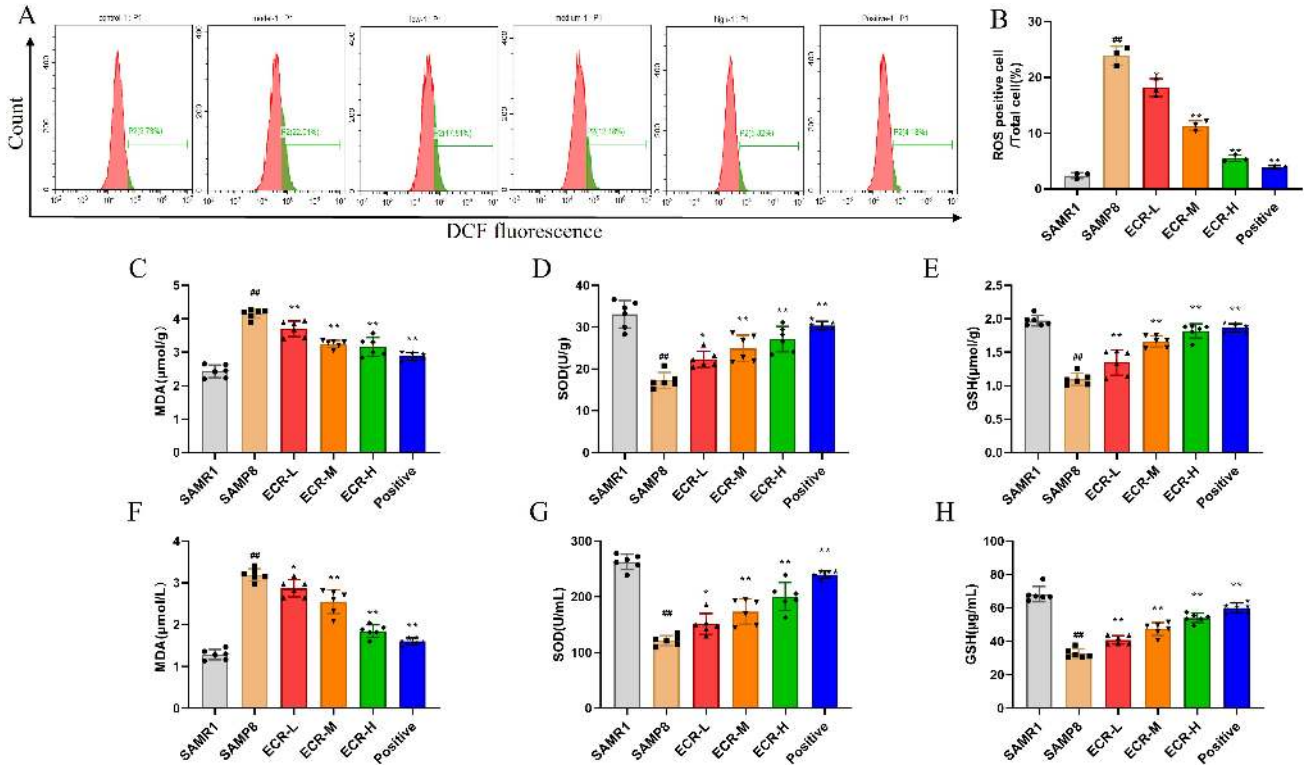


Fig. 3. Antioxidative effect of ECR against oxidative stress in SAMP8 mouse model. (A,B) Determination of levels of ROS in the hippocampus with flow cytometric technique after administration of different dosage forms (n = 3). (C) MDA, (D) SOD, and (E) GSH in the hippocampus were detected by biochemical kits (n = 6). (F) MDA, (G) SOD, and (H) GSH in serum were detected by biochemical kits (n = 6). The data were presented as the mean ± SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice. ROS, reactive oxygen species; MDA, Malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; DCF, 2′,7′-dichlorofluorescein.

group, however, ECR therapy may dramatically raise the Nrf2 expression level. As shown in Fig. 5D–H, western blot analysis revealed that ECR treatment could not only significantly increase P-Akt and P-GSK3β expression levels in the hippocampus of SAMP8 mice, but also markedly increased the hippocampal expression levels of Nucleus-Nrf2 and the Nrf2-related protein HO1. However, compared with the model group, ECR treatment significantly reduced the expression level of Cytosolic-Nrf2 (Fig. 5I). These findings suggest that ECR may improve cognitive impairments in SAMP8 mice via activating Akt/GSK3β to regulate Nrf2 and prevent oxidative damage.

3.5 Protective Effect of ECR on Oxidative Stress of Neurons in Aβ25-35-induced PC12 Cells

After demonstrating the potential of ECR to alleviate cognitive impairment via prevention of oxidative stress and death of neurons *in vivo*, we conducted several *in vitro* experiments to investigate the probable mechanistic action of ECR in cognitive impairment improvement. First, we screened the cell use concentrations of Aβ25-35 and ECR by MTT assay (**Supplementary Fig. 2**). We cultured PC12 cells and established an AD model treated with 25 μM Aβ25-35. Then we observed the effect of ECR on cell vitality by MTT, and the results showed that compared with the control group, the cell vitality decreased significantly after Aβ25-35 stimulation, but the cell vitality was reversed after ECR administration (Fig. 6C). Excessive ROS mediated antioxidant stress system imbalance is thought to be related to the AD process. Therefore, we investigated the ECR effect on oxidative stress in the Aβ25-35-induced AD model in PC12 cells with flow cytometric technique. Findings (Fig. 6A,B) of the above experiment showed that levels of ROS in model mice increased markedly compared to control, but their levels dose dependently decreased in mice that received ECR. Additionally, PC12 cells stimulated with Aβ25-35 showed alterations in SOD and GSH levels as well as an increase in MDA levels, with ECR reversing these modifications in the same manner as stated above (Fig. 6D–F). The expression levels of proteins associated with the Nrf2 antioxidant system and the levels of Cytosolic-Nrf2 and Nucleus-Nrf2 were detected using western blot to determine if the suppression of ECR on oxidative stress in Aβ25-35-induced PC12 cells *in vitro* is connected to the Nrf2 antioxidant system. Fig. 6G–M showed the results of western blot and cellular immunofluorescence analysis. We discovered that ECR activated the

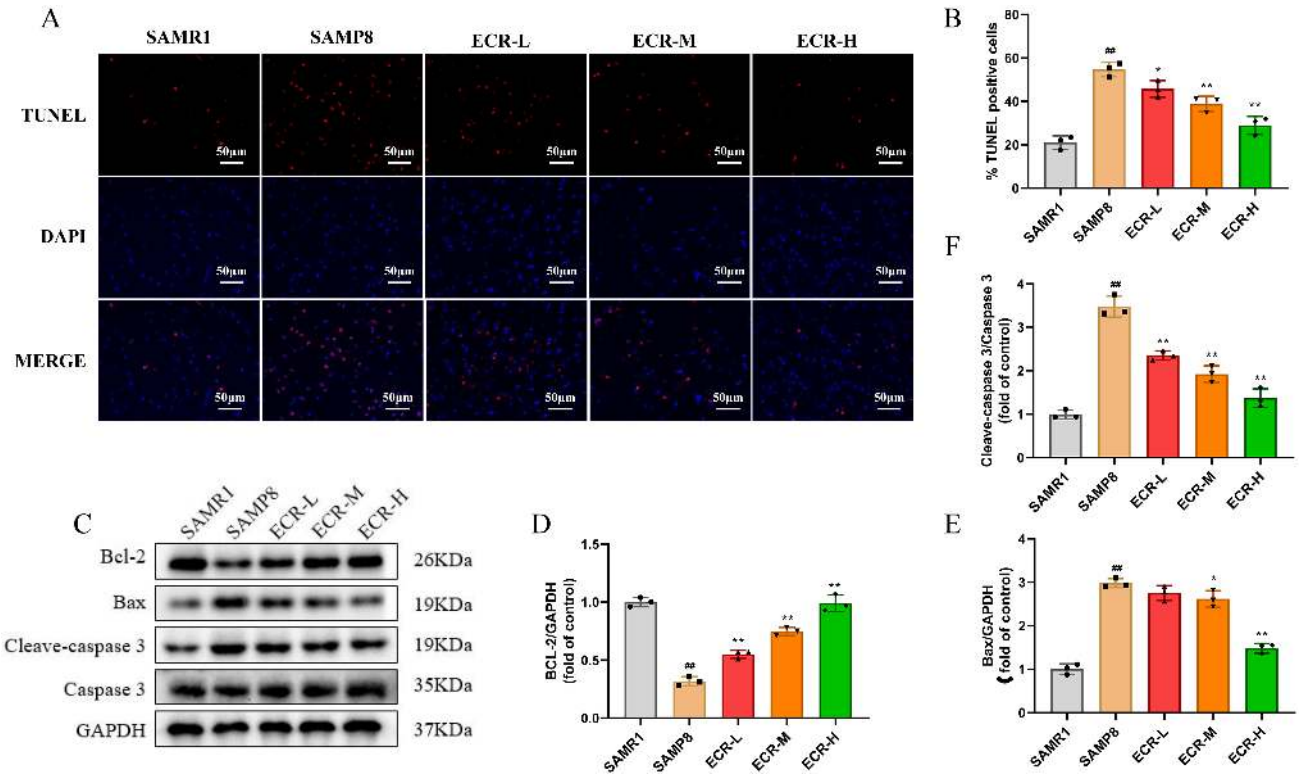


Fig. 4. ECR inhibits neuron apoptosis in SAMP8 mice. (A) Representative images of TUNEL staining in each group. (B) Apoptotic cells were quantitatively analyzed. In each group, n = 3, while the scale bar = 50 μm. (C) Detection of apoptosis-linked proteins, namely Bax, Bcl-2, cleave caspase-3, and caspase-3 in the hippocampus with western blotting technique. (D,E) Normalization of quantified levels of protein to GAPDH (n = 3). (F) Normalization of the quantified level of protein to caspase-3 (n = 3). The data were presented as the mean ± SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Nrf2 system, which substantially increased the expression of Nrf2 at the protein level in the nucleus.

In addition, we observed that PC12 cells treated with Aβ25-35 had an increased apoptotic rate and an increased expression of apoptosis-related proteins. In light of the flow cytometry result, the proportion of apoptotic cells in PC12 increased under the intervention of Aβ25-35 compared to control, while different doses of ECR significantly decreased apoptotic cells proportion in the above-mentioned cells (Fig. 6N,O). Furthermore, we evaluated the potential of ECR to prevent apoptosis in PC12 cells with western blot. Protein expression of Bcl-2 was found to be downregulated in PC12 cells stimulated with Aβ25-35, whereas the levels of caspase-3 and Bax were upregulated. ECR treatment ably upregulated Bcl-2 expression at protein level but downregulated Bax and caspase-3 protein expressions compared to PC12 cells in the model group (Fig. 6P–S).

Collectively, ECR demonstrated a beneficial effect on oxidative stress and cell apoptosis accordingly induced in PC12 cells by Aβ25-35.

3.6 ECR Regulates Nrf2 by Activating the Akt/GSK3β Pathway to Protect Cell Damage of Aβ25-35-induced PC12 Cells

During this experiment, we employed Akt inhibitor MK2206 to further clarify the possibility that ECR protected PC12 against oxidative stress and apoptosis induced by Aβ25-35 via regulation of Nrf2 by the Akt/GSK3β pathway. On the basis of the finding (Fig. 7A), we identified that the declined cell viability in Aβ25-35-induced cells was ameliorated by ECR, but inhibition of Akt caused this effect to disappear. Similarly, ECR treatment alleviated intracellular ROS levels in Aβ25-35-induced PC12 cells, but this phenomenon could also be eliminated by inhibiting Akt (Fig. 7B). Additionally, as depicted in the western blot results presented in Fig. 7C, ECR treatment increased the expression levels of P-GSK3β and Nucleus-Nrf2, while decreasing the expression level of Cytosolic-Nrf2 in Aβ25-35-induced PC12 cells. However, these changes would disappear due to the inhibition of Akt. Collectively, these findings provide further evidence that ECR controls Nrf2 by activating the Akt/GSK3β pathway to protect PC12 cells from damage induced by Aβ25-35.

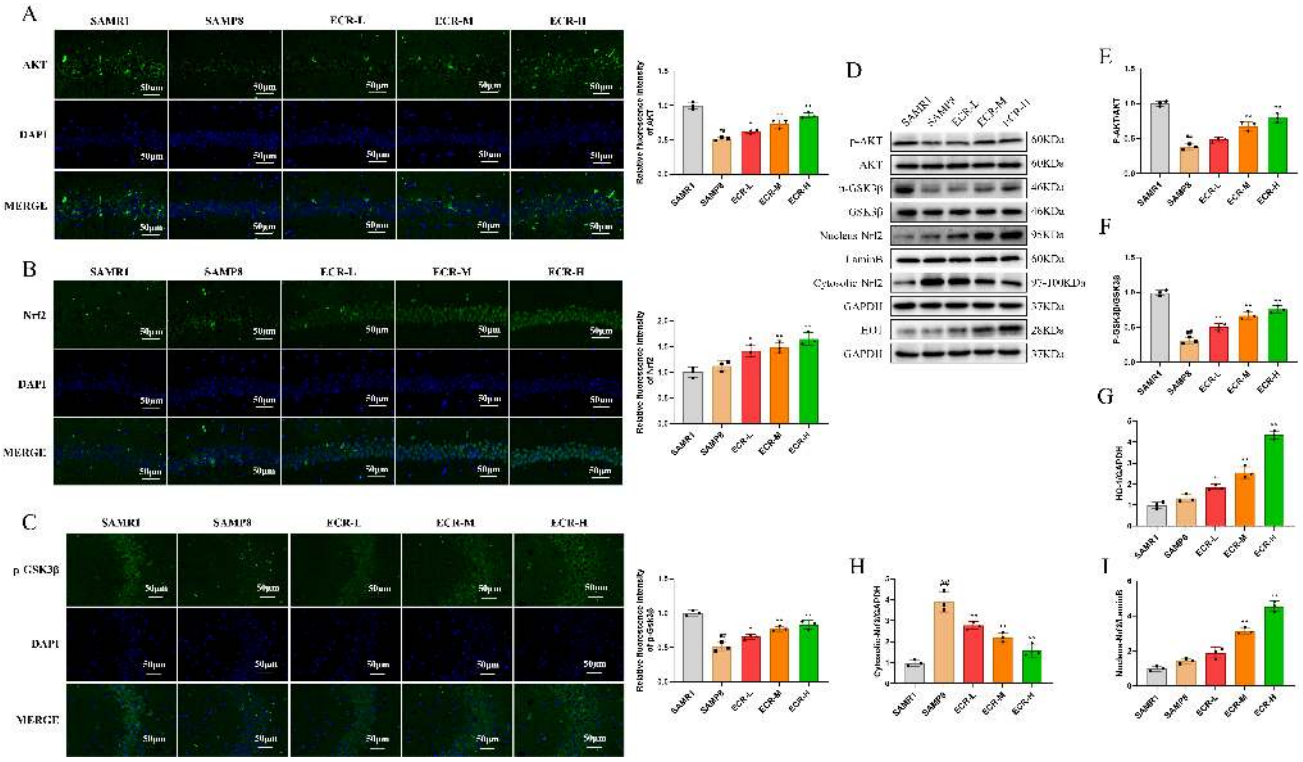


Fig. 5. ECR improves cognitive deficits in SAMP8 mice by activating Akt/GSK3β to regulate Nrf2. Detection of (A) Akt, (B) Nrf2 and (C) P-GSK3β expressions in the hippocampus with immunofluorescence staining. In each group, n = 3, while scale bar = 50 μm. (D) Analysis of Akt, P-Akt, P-GSK3β, GSK3β, nuclear-Nrf2, Cytosolic-Nrf2 and HO1 in the hippocampus with western blotting. (E) Normalization of P-Akt protein level to Akt (n = 3). (F) Normalization of P-GSK3β protein level to GSK3β (n = 3). (G) Normalize HO1 protein level to GAPDH (n = 3). (H) Normalize Cytosolic-Nrf2 protein level to GAPDH (n = 3). (I) Normalize Nuclear-Nrf2 protein level to LaminB (n = 3). The data were presented as the mean ± SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice. Akt, protein kinase B; Nrf2, nuclear factor erythroid-2-related factor-2; GSK3β, glycogen synthase kinase 3β; HO-1, heme oxygenase-1.

4. Discussion

Herein, we attempted to understand the mechanism underlying the potential of ECR to ameliorate cognitive impairment by conducting a series of experiments (*in vitro* and *in vivo*). Results obtained from SAMP8 animal models *in vivo* suggest that ECR may regulate Nrf2 to prevent oxidative stress in an Akt/GSK3β-dependent manner, thereby reducing cognitive impairment. Moreover, *in vitro* findings demonstrated that ECR regulates Nrf2 via the Akt/GSK3β pathway, protecting PC12 cells from Aβ25-35-induced cell damage. These findings collectively provide initial evidence that ECR could reduce cognitive impairment via the prevention of oxidative stress, amid regulation of Nrf2 via the Akt/GSK3β pathway.

The leading cause of dementia and a growing global health concern, AD has significant effects on both individuals and society [29]. Despite significant research efforts to find a therapeutic approach to stop the course of AD or to cure it, currently available medications are only effective in treating its symptoms and their efficacy is still insufficient [5]. Hence, efficacious treatment options for AD are urgently needed. ECR is a natural substance and the main steroid hormone. It has anti-oxidative and neuroprotective properties [22,30]. However, the therapeutic impact of ECR on AD and its molecular mechanism have not been sufficiently investigated. The SAMP8 mouse model's cognitive deficiencies were found to be improved by ECR and positive treatment in this study, and ECR was demonstrated in a dose-dependent manner.

Oxidative stress, which is caused by an excess of ROS, has been associated with various disorders. Due to the high oxygen consumption in the brain, these free radicals may cause more visible damage than what cell molecules can scavenge [31]. Oxidative stress has emerged as a crucial approach in the prevention and treatment of AD since it plays a significant role in the disease progress and may be regarded as a major factor in its development [32–34]. Therefore, we evaluated the ECR effect on oxidative stress in the hippocampal of SAMP8 mice or PC12 celles induced by Aβ25-35. Our findings demonstrated that oxidative stress is activated in Aβ25-35-induced PC12 cells

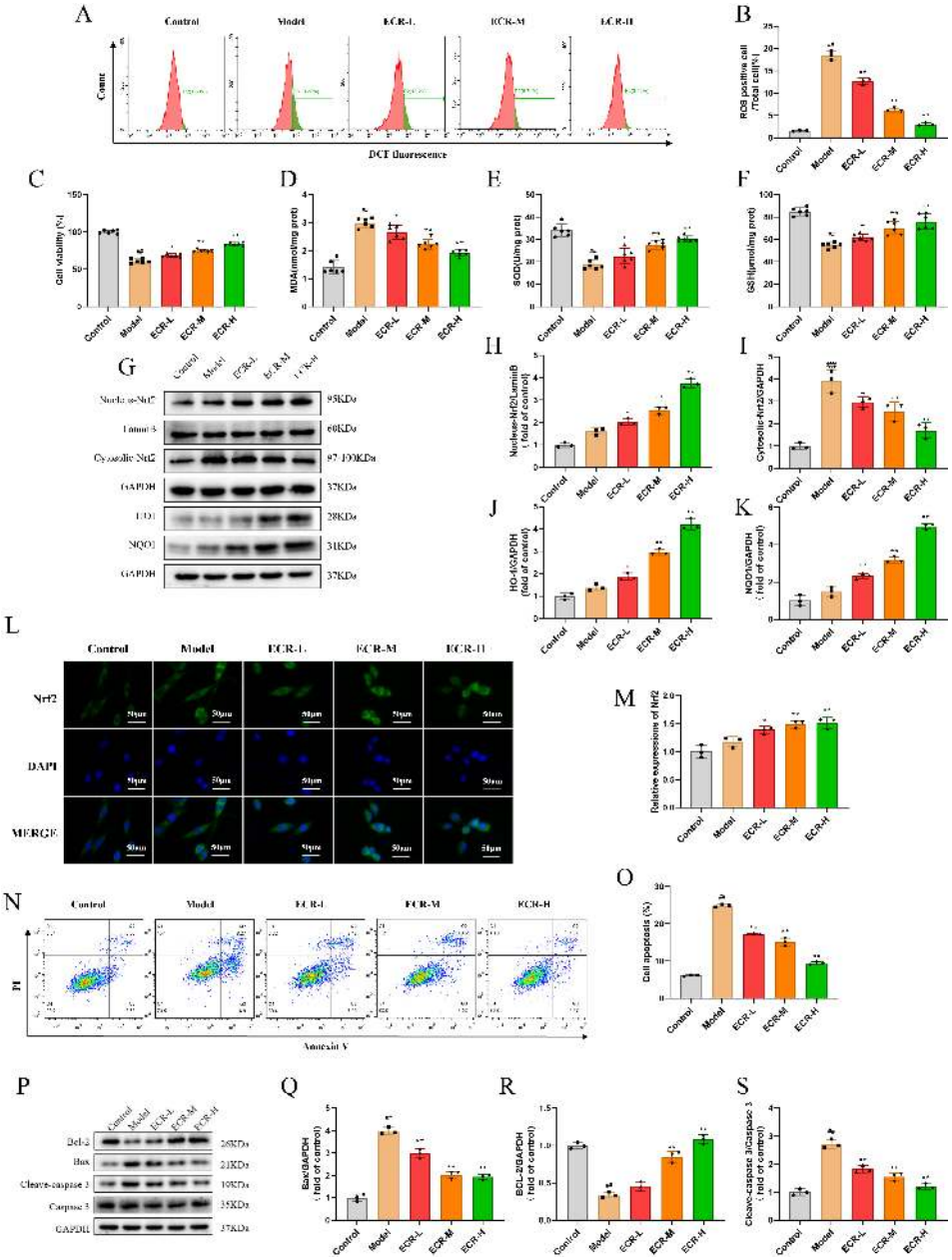


Fig. 6. Protective effect of ECR on oxidative stress of neurons in Aβ25-35-induced PC12 cells. (A) Determination of ROS levels in PC12 cells treatment with different dosage forms with flow cytometry. (B) Levels of ROS quantifications (n = 3). (C) Assessment of cell viability with MTT assay and treatment of Aβ25-35 induced PC12 cells with various doses of ECR (n = 6). Detection of biomarkers of oxidative stress (D) MDA, (E) SOD and (F) GSH in PC12 cells after treatment with different dosage forms (n = 6). (G) The expression levels of Nrf2 antioxidant system-related proteins HO1, NQO1, Cytosolic-Nrf2 and Nuclear-Nrf2 were analyzed by western blotting. (H) Normalize Nuclear- Nrf2 protein level to LaminB (n = 3). (I–K) Normalize HO1, NQO1 and Cytosolic-Nrf2 protein levels to GAPDH (n = 3). (L,M) Immunofluorescence staining analysis of Nrf2 expression in PC12 cells after treatment with different dosage forms. In each group, n = 3, while scale bar = 50 μm. (N,O) Determination of ECR effect on the percentage of apoptotic cells in PC12 cells induced by Aβ25-35 with flow cytometric technique (n = 3). (P) Western blotting analysis of Bax, Bcl-2, cleave caspase-3 and caspase-3 protein expressions in PC12 cells after different treatments. (Q,R) Normalization of levels of Bax and Bcl-2 protein to GAPDH (n = 3). (S) Normalization of the level of cleaved caspase-3 to caspase-3 (n = 3). The data were presented as the mean ± SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. The groups were compared as follows; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to model mice; $^{\#\#}p < 0.01$ compared to control mice. MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide; NQO1, NADH dehydrogenase (quinone 1).

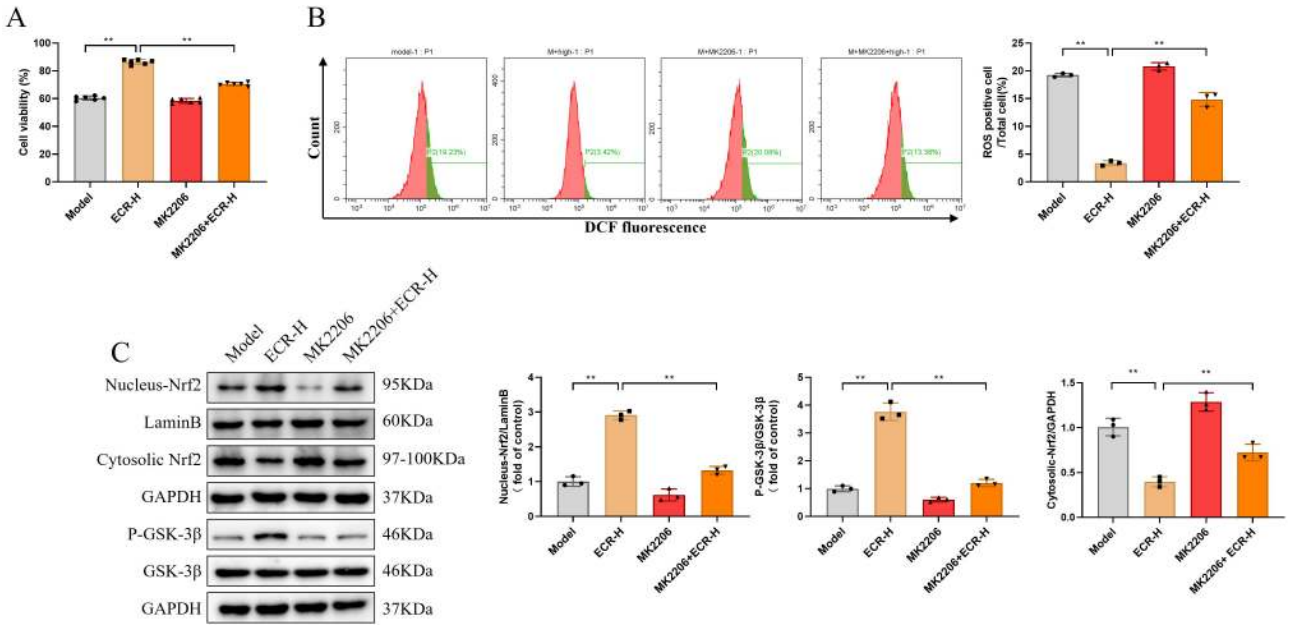


Fig. 7. ECR regulates Nrf2 by activating the Akt/GSK3β pathway to protect cell damage of Aβ25-35-induced PC12 cells. (A) Determination of the effect of high concentration of ECR on cell viability of PC12 cells induced by Aβ25-35 before and after Akt inhibitor treatment with MTT assay (n = 6). (B) Effect of high concentration of ECR on ROS levels in PC12 cells induced by Aβ25-35 before and after Akt inhibitor treatment was analyzed by flow cytometry (n = 3). (C) Western blotting analysis of the effects of high ECR concentration on levels of Cytosolic-Nrf2, Nuclear-Nrf2, P-GSK3β and GSK3β expressions in PC12 cells before and after Akt inhibitor treatment. Normalization of the level of nuclear-Nrf2 protein to LaminB (n = 3). Normalization of the level of P-GSK3β protein to GSK3β (n = 3). Normalize Cytosolic-Nrf2 protein level to GAPDH (n = 3). The data were presented as the mean $\pm$ SD after being subjected to one-way ANOVA and Tukey's post hoc test analysis. $^{**}p < 0.01$, means significant difference.

or SAMP8 model mice, but that ECR treatment increases SOD and GSH levels while decreasing ROS and MDA levels. This suggests that ECR may improve cognitive deficiencies by preventing oxidative stress. Additionally, AD patients frequently experience neuronal death in the brain, particularly in the hippocampus, which is largely brought on by apoptosis, with oxidative stress serving as the primary trigger [35]. As a result, we investigated how ECR affected apoptosis in PC12 cells induced by Aβ25-35 or in the hippocampus of SAMP8 mice. Our findings suggested that ECR, particularly in the high-dose group, attenuates the decline in Bcl-2 and the rise in Bax and Cleave-caspase 3 protein expression in the hippocampus of SAMP8 mice. Similar results were observed for Aβ25-35-induced PC12 cells. Based on the above *in vivo* and *in vitro* results, we suggested that ECR has an anti-apoptotic potential in AD, probably through inhibition of oxidative stress.

Apart from crucially regulating the antioxidative system, Nrf2 has been found to additionally control the response of inflammation, homeostasis of intracellular redox and other processes of biological importance [36]. Besides, research has demonstrated that one of the body's most crucial antioxidant defense mechanisms is the Nrf2/HO1 pathway [37]. It has been posited that the antioxidative system of Nrf2 (for example) might reduce the severity of several diseases by preventing oxidative stress when downstream genes like NQO1 and HO1 are controlled [38]. Additionally, one of the several upstream signaling pathways that target Nrf2 is the Akt/GSK3β pathway [16]. GSK3β regulates cell survival activity as a critical factor downstream of Akt, and Akt-mediated phosphorylation of GSK3β inhibits its expression [39]. Of note, drugs that possess inhibitory action against GSK3β have demonstrated potential pharmacological treatments for AD, since they could lessen CI and neuropathological symptoms *in vivo* [40]. Therefore, we verified the ECR effect on Nrf2 and Akt/GSK3β pathway. Research conducted both *in vitro* and *in vivo* has demonstrated that ECR can powerfully enhance Akt and GSK3β phosphorylation levels, as well as boost Nucleus-Nrf2 protein expression and enhance its function within the nucleus. The increased HO-1 and NQO1 expression levels found in this investigation offered more proof. Moreover, the current work evidenced through Akt inhibitor the potentiality of ECR to activate the antioxidative system of Nrf2 via the Akt/GSK3β pathway. The results showed that Akt inhibitors eliminated changes in cell activity, oxidative stress, and the expression of P-GSK3β and Nucleus-Nrf2 after ECR treatment. Collectively, these findings suggest that ECR can regulate Nrf2 via the Akt/GSK3β pathway to attenuate oxidative stress-induced apoptosis, and thereby improve cognitive impairment.

5. Conclusions

In conclusion, this study suggested that ECR reduced oxidative stress by activating the antioxidative system of Nrf2 via the Akt/GSK3β pathway, thereby improving cognitive impairment. These findings suggest that natural medicine like ECR could act as an efficacious therapeutic intervention for AD. Given the increased incidence rate of AD, the applications of ECR in the medical field may be broadened. The target of ECR, however, was not investigated in depth, which is a shortcoming of this study. We'll look at this issue more in the next research.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

HX wrote the original draft. HX, SX and DC designed the research study. HX and GC performed the research. FR and HZ provided help and advice on the experiments. LX, QL and RL analyzed the data. HX and DC acquired the funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The Jiangsu University (UJS IACUC) institutional committee for the care and use of laboratory animals reviewed and approved (approval number: UJS-IACUC-2022031401) for studies involving animals.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2903109.

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