# Isorhamnetin improves cognitive impairment of Alzheimer's disease through Sirt1/AKT/ERK/CREB signaling pathway

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**Research Article**

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# Abstract

Isorhamnetin (IH) is one of a flavonoid with extensive pharmacological activities, including anti-inflammation and antioxidation. Although IH has a protective effect in neurological diseases, its role in Alzheimer's disease (AD) is not very clear. In order to explore the effect and mechanism of IH in AD, 4-month-old APP/PS1mice treated with IH in 50 mg/kg/d for 3 months and Morris water maze and immunofluorescence staining were used to detect whether IH could improve AD pathology. The mechanisms of IH improving AD pathology were detected by Western blot. The results showed that after IH treatment, the time of escape latency was significantly reduced and the number of crossing the platform was significantly increased, suggesting that IH can improve the cognitive impairment of mice. Moreover, IH significantly decreased senile plaques deposition in the brain and increased the expression of ADAM10 in APP/PS1 mice through activating Sirt1/AKT/ERK/CREB pathway. Furthermore, IH reduced ROS formation, GP91 expression, upregulated the expression of SOD1 and Nrf2. Moreover, IH inhibited astrocytes activation and pro-inflammatory cytokine IL-1β and TNF-α release. In conclusion, IH improved cognitive function, inhibited oxidative stress and inflammation response in AD. Therefore, we suggest that IH may be considered as a potential drug for AD treatment.


# Introduction

Alzheimer's disease(AD) is a neurodegenerative disorder with insidious onset and slow progression and with the increasing incidence of this disease, the medical and social burden are increasingly serious [1–2]. Although five drugs have been approved for AD, the effect of these drugs is to improve symptoms rather than delay the pathological process of AD [2]. The main reason is that the pathogenesis of AD is not clear. There are many hypotheses about the pathogenesis of AD and the amyloid hypothesis is considered to be the major one. Amyloid-beta (Aβ) is produced by amyloid precursor protein (APP) via sequential proteolytic cleavages through β-secretase (BACE1) and γ-secretase [3]. The aggregation of Aβ will cause a series of pathological changes, such as synaptic loss, neuroinflammation, neuronal death and so on. In addition, the pathway of APP cleaved by the α-secretase A Disintegrin And Metalloprotease 10 (ADAM10) releases the soluble portion (sAβPPα) which could prevent generation of Aβ [3]. Therefore, ADAM10 can be used as a target for the treatment or prevention of AD [3].

Isorhamnetin (IH), as one of the active components from the fruits of Hippophae rhamnoides L. and the leaves of Ginkgo biloba L., has a wide range of pharmacological activities, such as anti-inflammatory, anti-oxidation, etc [4]. It has been showed that IH protected human RPE cells against $H_2O_2$-induced oxidative stress and cell death through activating the PI3K/Akt signaling pathway [5]. IH can also significantly improve high-fat and high fructose diet (HFFD)-induced cognitive impairments, which is due to attenuate HFFD-induced microglial activation and inflammatory cytokines released via inhibiting the MAPK and NF-κB signaling pathways [6]. In addition, IH relieved the high glucose (HG)-induced oxygen-glucose deprivation and reoxygenation (OGD/R)-induced apoptosis, inflammatory response, and oxidative stress of HT22 cells via Akt/Sirt1/Nrf2/HO-1 pathway [7]. Recent research shows that IH treatment can promotes functional recovery in rats induced by spinal cord injury via inhibiting oxidative

stress and modulating M1/M2 polarization of macrophage [8]. The findings of the above studies suggested that isorhamnetin may have potential benefits in antioxidative effect and treatment of oxidative damage diseases. However, the effect of isorhamnetin on AD is not clear. Therefore, in this study, we investigated the effect of IH in improving cognitive impairment and its mechanism in AD model mice.

## Materials And Methods

# Animals and treatment

In this study, 4-month-old male APP/PS1 transgenic were randomly divided into two groups: vehicle and IH groups. In IH group, IH with a dose of 50 mg/kg/d was injected intraperitoneally once a day for 3 months, and the vehicle group was given the same dose of solvent. Mouse were kept the standardized conditions. All experimental procedures performed using animals were approved by the Laboratory of Animal Ethical Committee of China Medical University.

# Behavior Tests

Morris water maze was used to detect the behavior of APP/PS1mice after 3 months treatment. First, the mice were trained on the visual platform for two days, and then conducted the hidden platform experiment for five days to record the latency time of mice finding the platform by the water maze system (ZH0065; Zhenghua Bioequipment, China). On the eighth day, the platform was removed and the number of mice crossing the platform was recorded.

# Tissue preparation

The mice were anesthetized and perfused with PBS. After that, the brains were quickly collected on ice, and one hemisphere was frozen and stored at -80°C, and the other was immersion-fixed in 4% paraformaldehyde for the histological study.

# Reactive oxygen species (ROS) assay

Fresh tissue of the brain was prepared cell suspension and then centrifugation. Cell precipitation was resuspended with diluted DCFH-DA and incubated at room temperature for 30 min. After washing with PBS for three times, and the fluorescence intensity of ROS was detected by Flow cytometry.

# Measurement of oxidative stress related enzymes activities

Brain tissue was added with cold PBS and homogenized at 4°C, and then centrifuged at 10000g at 4°C for 3–5 minutes. The protein concentration of the supernatant was measured by BCA protein assay kit, and then the activities of SOD and GSH were detected by assay kit according to the manufacturer's instructions (Najing Jiancheng).

# Immunofluorescence staining

The frozen sections were blocked with 5% goat serum for 30 min and incubated with anti-GAFP antibody and anti-Aβ overnight at 4°C. The sections were washed with PBS for 3 times and then incubated with Alexa Fluor 594- or Alex Fluor 488-conjugated secondary antibodies for 1.5 h. Images were detected by using the laser scanning confocal microscope (TCS SP8, Leica, Germany).

# Western blot

The brain tissues were homogenized with RIPA lysis buffer including protease and phosphatase inhibitor on ice for 3 h. After centrifugation, the protein concentration was determined through BCA protein assay kit. The protein samples were loaded according to the same amount of protein and were separated by 10% SDS-PAGE gels. After transfection, PVDF membrane was blocked with 5% BSA for 30 min and incubated with different primary antibodies overnight at 4°C. The next day, membranes were washed 3 times with TBST and then incubated with HRP-conjugated secondary antibody for 1.5 h, and finally were detected using ECL.

# Results

### IH treatment significantly improve cognitive impairment in APP/PS1 mice

To illustrate whether IH improves cognitive impairment, APP/PS1 mice were subjected to Morris water maze. The results showed that after IH treatment, the latency time for mice to find the platform was significantly reduced (Fig 1A). The number of mice crossing the platform was significantly more than that of the vehicle group (Fig 1B). The results suggested that IH could significantly improve the cognitive impairment of mice.

### IH treatment alleviated Aβ deposition in APP/PS1 mice

In order to explore the mechanism of IH improving behavior, we detected the generation of senile plaques by immunofluorescence. The results showed that the senile plaques in the brain of mice decreased significantly after 3 months of IH treatment (Fig2).

### IH up-regulated the expression of ADAM10

Aβ is produced by the cleavage of APP through amyloid pathway, BACE1 and γ-secretase are the main cleavage enzymes. The results showed that IH had no effect on the expression of BACE1 and γ-secretase (Fig 3A-B). However, in this study, we found that ADAM10, as the main enzyme in the non-amyloid pathway, can be significantly up regulate its expression by IH (Fig 3A-B) and then reduce the production of Aβ.

### IH inhibited oxidative stress in APP/PS1 mice

Oxidative stress plays an important role in the pathogenesis of AD. Over-production of reactive oxygen species (ROS) and disorder of antioxidant capacity result in subsequent oxidative damage [9]. To illustrate the effects of IH on oxidative stress, we investigated the levels of ROS, SOD and GSH in brain

tissue of mice. The results showed that the fluorescence intensity of DCFH-DA was significantly decreased after IH treatment (Fig 4A). The level of SOD and GSH was enhanced compared to the vehicle group (Fig 4B).

gp91 has been recognized as biomarkers of oxidative stress [10]. In this study, IH significantly decreased the level of gp91 (Figure 4C). Taken together, these results illustrated that IH could alleviate oxidative stress in APP/PS1 mice.

A large number of studies have shown that the transcriptional factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) plays an important defensive role in the antioxidant response of the brain by promoting the expression of several antioxidant enzymes [11]. In our paper, we found that IH increased the expression of Nrf2 to against oxidative stress (Fig 4C).

### Isorhamnetin reduced inflammatory response in APP/PS1 mice

To confirm if IH could inhibit inflammatory response in AD, we detected the release of inflammatory factors and astrocytes activation. The results found that after IH treatment, the expression of TNF-α and IL-1β were decreased (Fig 5A-B). Western blotting and immunofluorescence results showed that IH could inhibit astrocytes activation induced by Aβ (Fig 5B).

### Isorhamnetin activated Sirt1/AKT/ERK/CREB signaling pathways

Previous study showed that CREB, the promoter of ADAM10, could up- regulate CREB and promote the expression of ADAM10 [12]. ERK is the upstream of CREB, it can be activated by Akt and its activation can promote the phosphorylation of CREB [12]. Our results showed the IH significantly promoted phosphorylation level of Akt, ERK and CREB (Fig 6A-B). Notably, expression of Sirt1 activated the Akt signaling pathway, and IH could increase the Sirt1 expression (Fig 6A-B).

## Discussion

The results of this study indicate that IH, a flavonoid, can significantly improve the cognitive impairment of APP/PS1 transgenic mice, reduce the deposition of senile plaques in the brain, up-regulate the expression of ADAM10 by activating SIRT1/Akt /ERK/CREB signal pathway and promoting the non-amyloid pathway of APP. Moreover, IH reduced the production of ROS in the brain, increased the expression and activity of antioxidant enzymes SOD1, GSH and the expression of Nrf2, inhibited inflammation, and improved the pathological process of AD.

Aβ is produced from APP through sequential cleavages by BACE-1 and γ-secretase [13]. In nonamyloidogenic pathway, APP can be cleaved by α-secretase and γ-secretase to prevent production of Aβ [3]. ADAM10 (metalloprotease) is considered the major α-secretases in neurons [13]. Up-regulating ADAM10 can suppress Aβ production [14], suggesting that ADAM10 may serve as a potential target in AD treatment. Previous study showed that CREB, induced as ADAM10 promoter, could increase the expression of ADAM10 to preclude Aβ production [12]. The ERK-CREB signal pathway has been studied

for its role in AD [15]. Previous studies have shown that the neuroprotective effect of AD may be caused by CREB activation mediated by ERK activation, which promotes the expression of ADAM10 [15]. It has been showed that up-regulating ERK/CREB pathway can resist Aβ1-42-induced damage [16]. Our results showed that IH promoted the phosphorylation level of ERK and CREB. According to these results, IH plays the protective effect in AD via promoting ADAM10 by activating ERK/CREB pathway.

Previous studies have shown that the activation of Akt phosphorylation plays a protective role in AD and activated Akt signaling pathway can up regulate the expression of HO-1 and Nrf2 [17–18]. The phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathway activation could prevent Aβ deposition in brain and reduce Aβ-induced neurotoxicity in primary neuronal [19]. In addition, as an upstream kinase regulating ERK activation, Akt inhibitor or Akt siRNA could reduce the phosphorylation level of ERK [20], suggesting that ERK phosphorylation is dependent on Akt. Sirt1 as one of $NAD^+$-dependent deacetylase, decreased amyloidogenic cleavage of APP by increasing ADAM10 activity in AD [21]. Moreover, Sirt1 can mediate the Akt activation [22]. In our study, we found IH could activate Akt via increasing Sirt1 expression and then led to upregulate the ERK phosphorylation.

Oxidative stress involved in the development of AD through increasing Aβ generation and tau protein phosphorylation level, eventually lead to neuronal death. Therefore, oxidative stress is considered to be an important part of the pathological process of AD [23]. gp91 had been recognized as biomarkers of oxidative stress [10]. Our results showed that IH treatment could reverse the up-regulation of ROS and gp91 expression. Moreover, the activities and expression of antioxidant enzymes SOD and GSH were increased. Nrf2 participates in antioxidant defense process by restoring the decreased activities of GSH, CATs and SODs [24–27]. It has been showed that up-regulating Nrf2 could improve $H_2O_2$-induced oxidative stress damage [24]. Our results illustrated that IH treatment enhanced the Nrf2 expression, thereby improving the activity of antioxidant enzymes.

Inflammation is also associated with AD and that could have a vital role in contributing to the pathogenesis of AD [28]. As one of the most common glial cells, astrocytes play an important role in neuroinflammation in the brain by releasing inflammatory cytokines, including tumor necrosis factor alpha, interleukins, etc [29–30]. Our results show that IH can inhibit the activation of astrocytes and the release of inflammatory factors and reduce the inflammatory response.

In conclusion, IH promotes the expression of ADAM10 by activating Sirt1/Akt/ERK/CREB signaling pathway, thereby reducing the deposition of senile plaques. In addition, IH can also inhibit oxidative stress and inflammatory response. finally, improve the cognitive impairment of AD.

# Declarations

### Ethics approval

All experimental procedures performed using animals were approved by the Laboratory of Animal Ethical Committee of China Medical University.

**Consent to Participate** Not applicable

**Consent to Publication** Not applicable

### Data Availability

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

### Competing interests

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.


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There is no funding.


### Authors Contributions

Ying Li and Sha Sha designed research and wrote the paper. Zhang Rong Wei participated in data analysis and Figures making. All authors have read and approved the last manuscript.


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# Figures

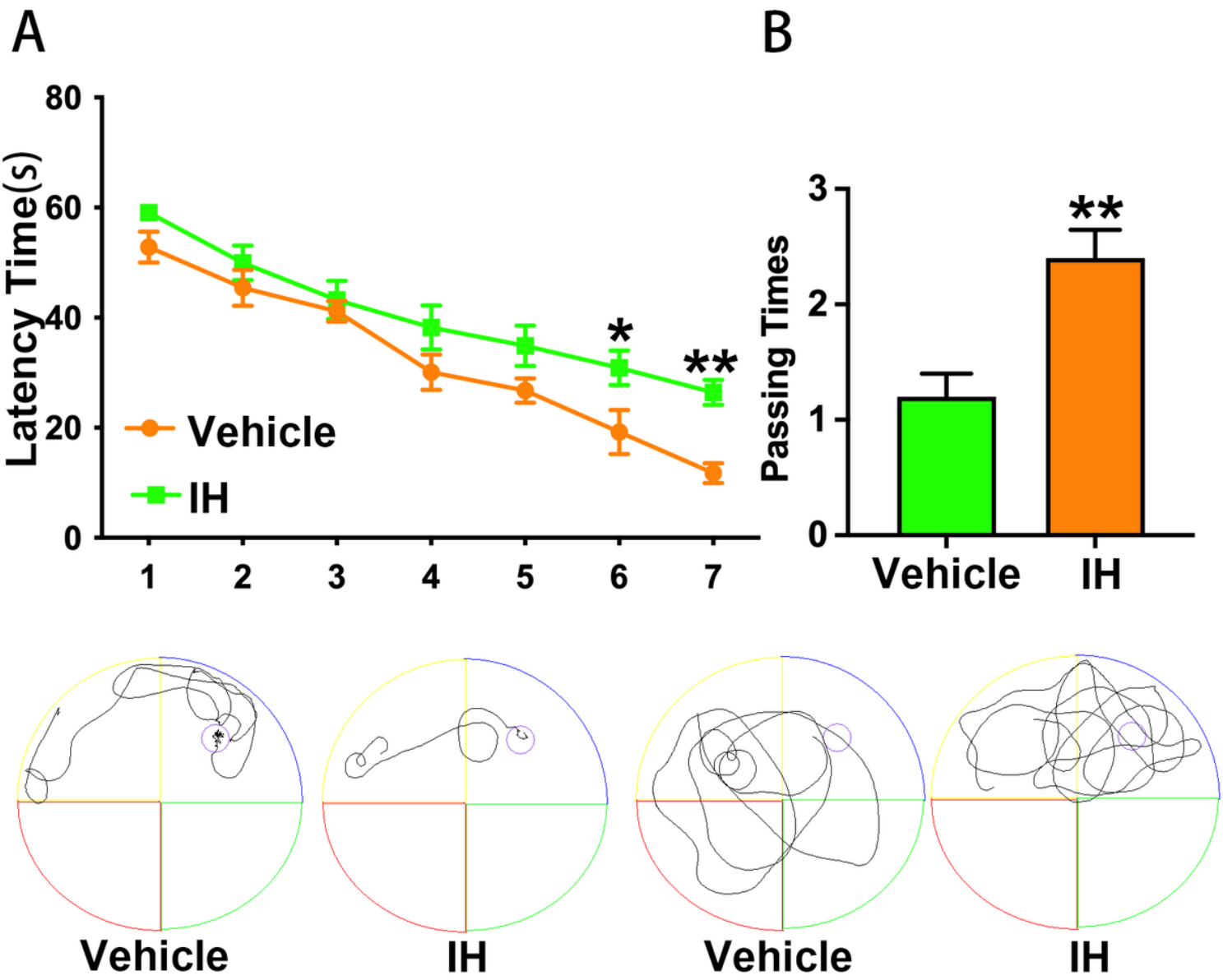
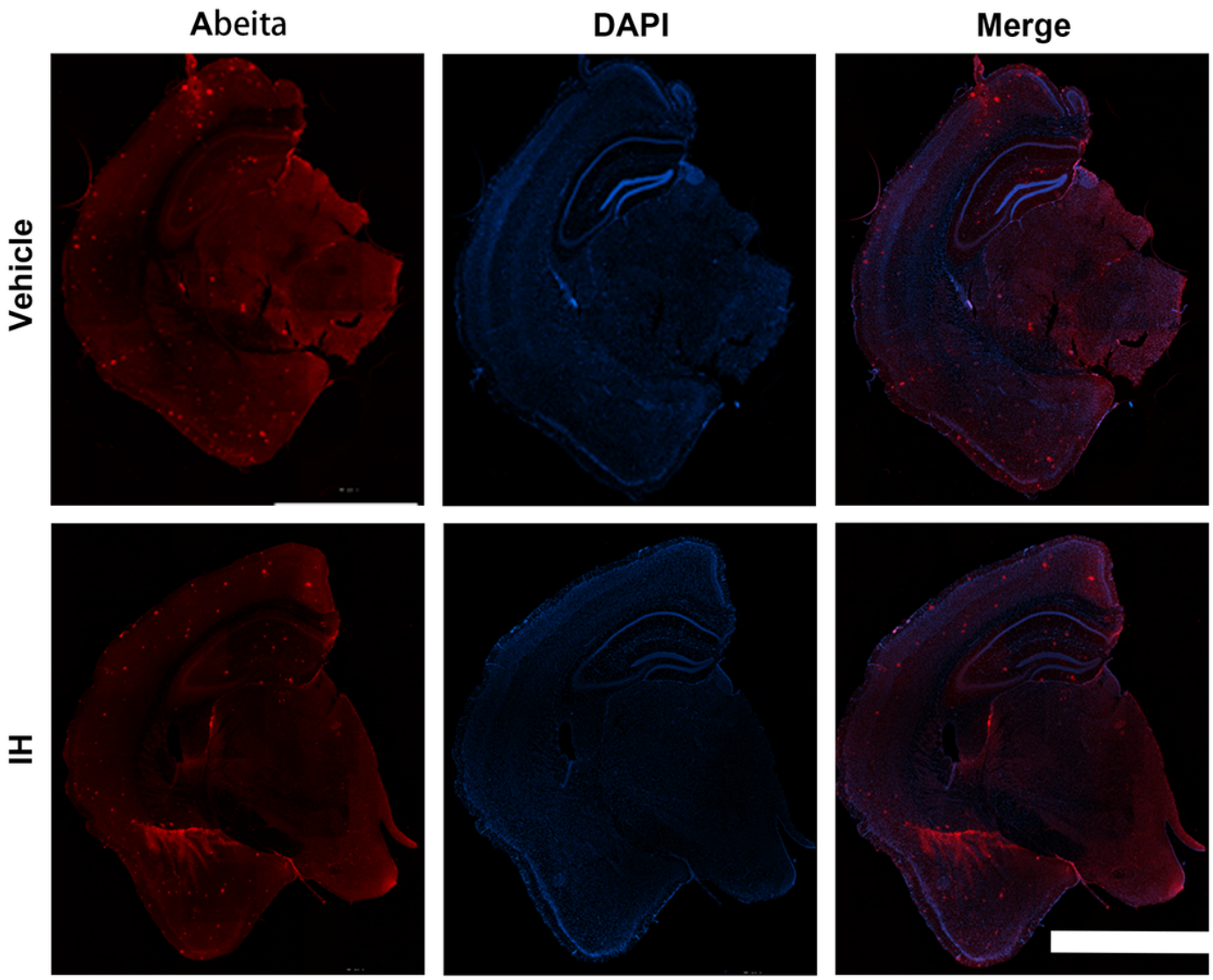


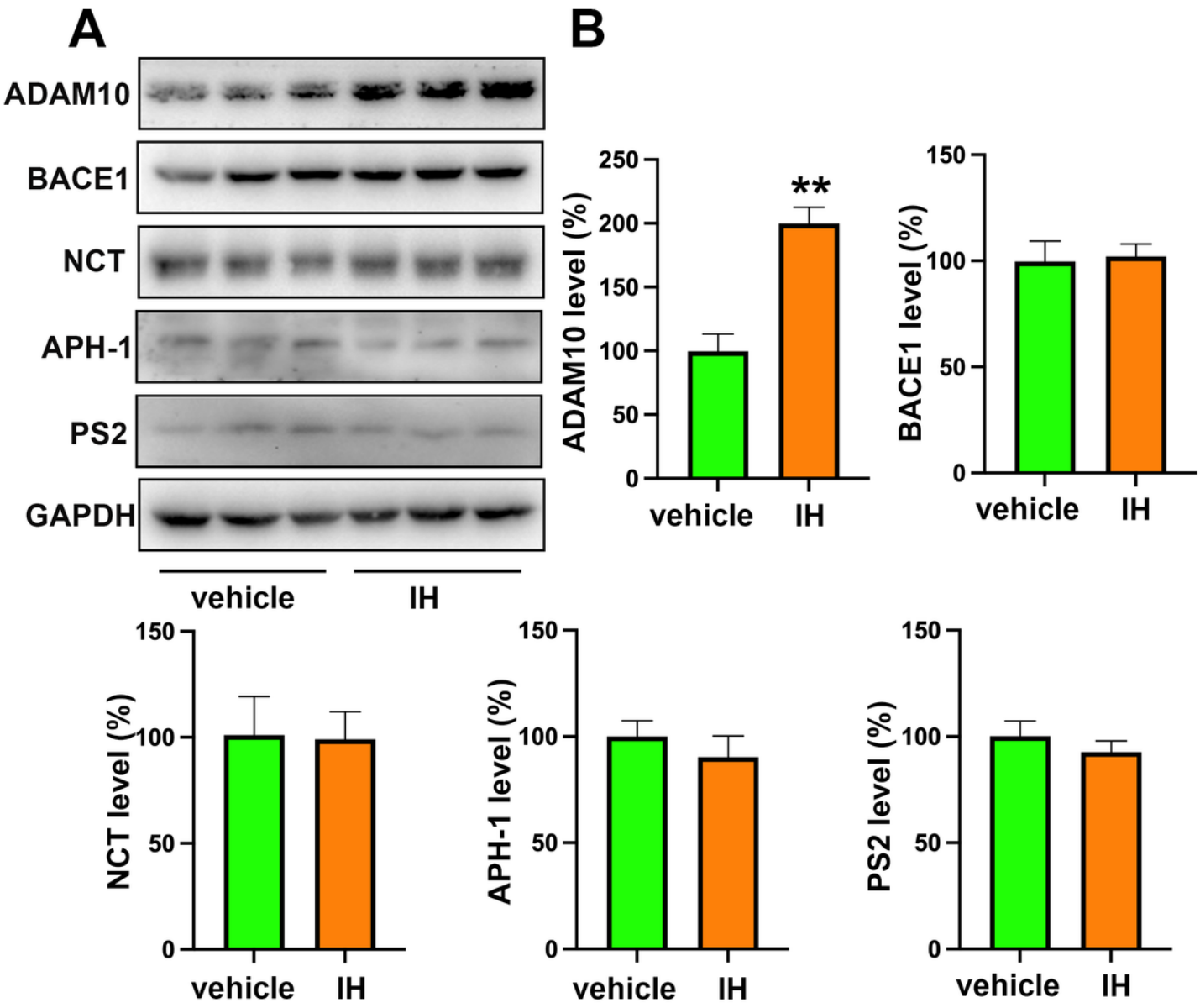

**Figure 1**

IH **improves cognitive impairment of APP/PS1 mice. Four-month-**APP/PS1 mice were treated with IH (i.p., 50 mg/kg/d) for 3 months. Morris water maze was used to test cognitive ability including 2 days of visible platform training, 5 days of hidden platform testing, and a probe trial. (A) In the visible platform and the hidden platform, the escape latency time of mice found the platform. (B) In the probe trial, the times of crossing the platform were recorded. Data were presented as the mean ± SD; n = 6, * $P < 0.05$; ** $P < 0.01$.



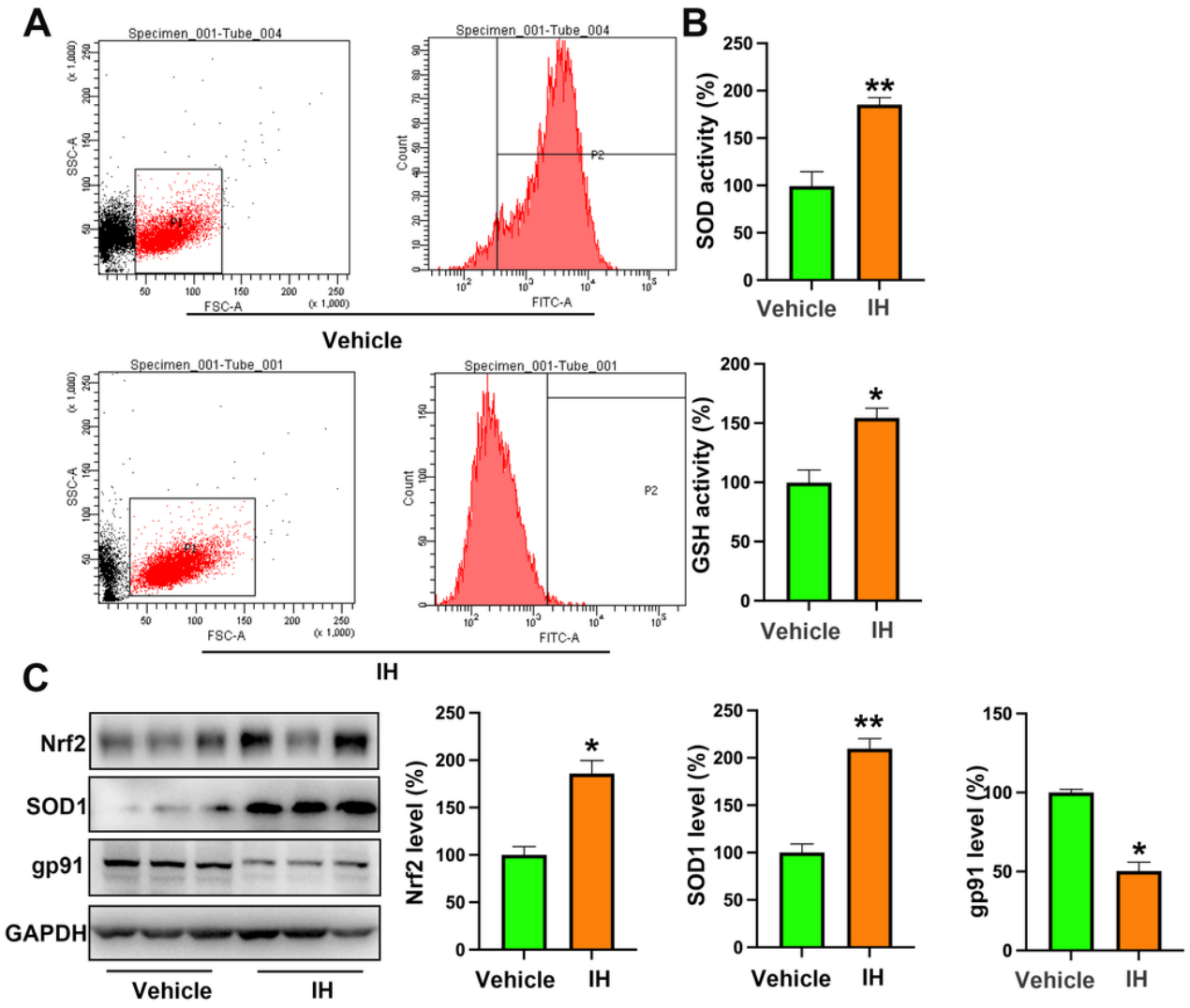

## Figure 2

**IH inhibites senile plaque depostion in APP/PS1 mice.** Four-month-old APP/PS1 mice were treated with IH for 3 months. Aβ plaque in the cortex and hippocampus of APP/PS1 mice were detected by immunofluorescent.



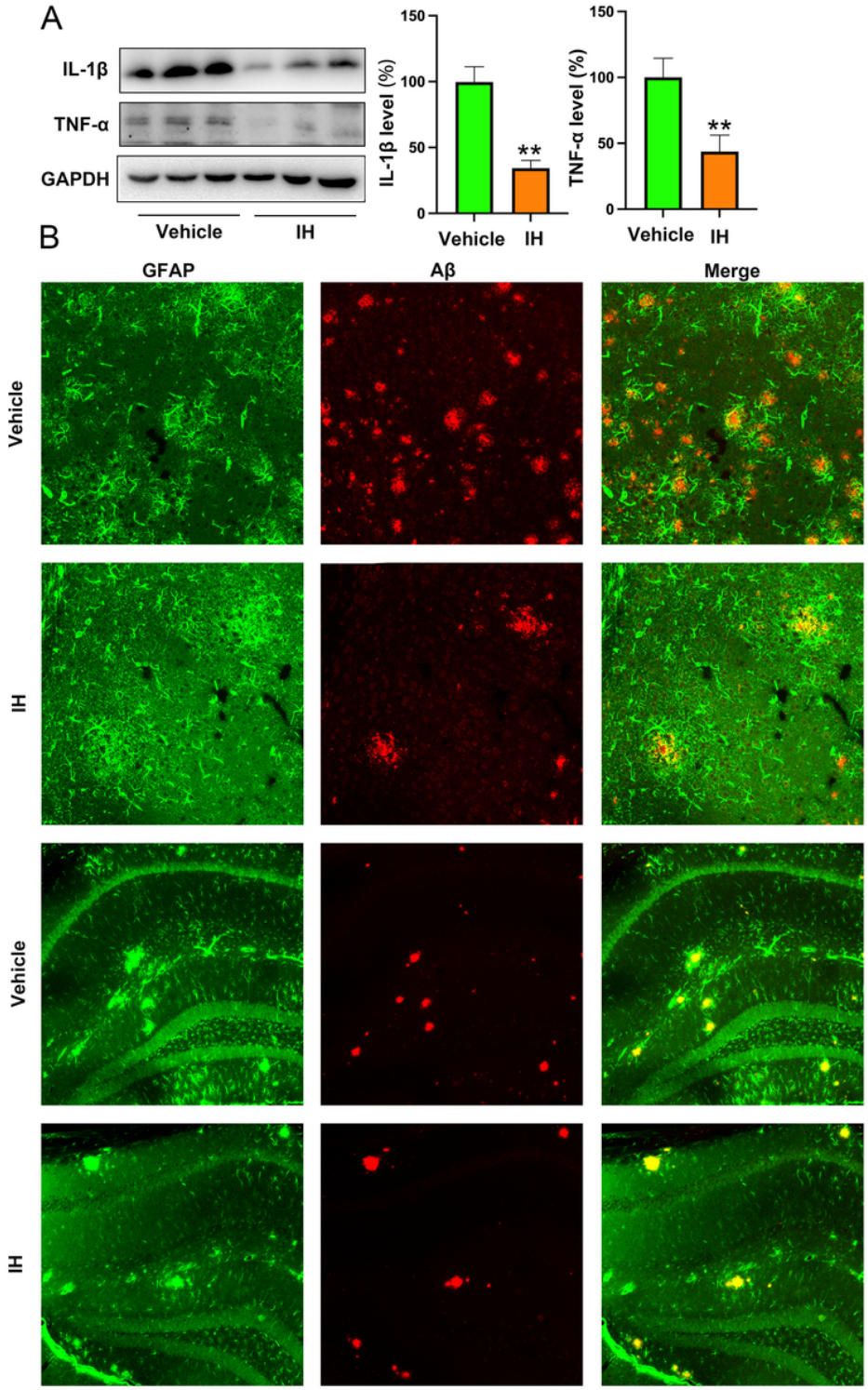

## Figure 3

**IH upregulates the expression of ADAM10 in APP/PS1 mice.** Four-month-old APP/PS1 mice were treated with IH for 3 months. Western blot detect the expression levels of ADAM10, BACE1 and the subunits of γ-secretase, including NCT, PS2 and APH-1. Data were presented as the mean ± SD; n = 6, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.




## Figure 4

**IH reduce the oxidative stress in APP/PS1 mice.** Four-month-old APP/PS1 mice were treated with IH for 3 months. (A,) Western blot detect the expression levels of SOD1, Nrf2 and GP91 in the cortex. (B) ROS production in the cortex was detected with the dichlorofluorescein diacetate probe. Data were presented as the mean ± SD; n = 6, * $P < 0.05$; ** $P < 0.01$.




## Figure 5

**IH alleviated the neuroinflammatory response in APP/PS1 mice.** Four-month-old APP/PS1 mice were treated with IH for 3 months. (A-B) Immunoblot analysis showed the expression levels of IL-1β and TNF-α. (C) IH treatment suppressed the activation of astrocytes around the Aβ plaque. Data were presented as the mean ± SD; n = 6, ** $P < 0.01$.

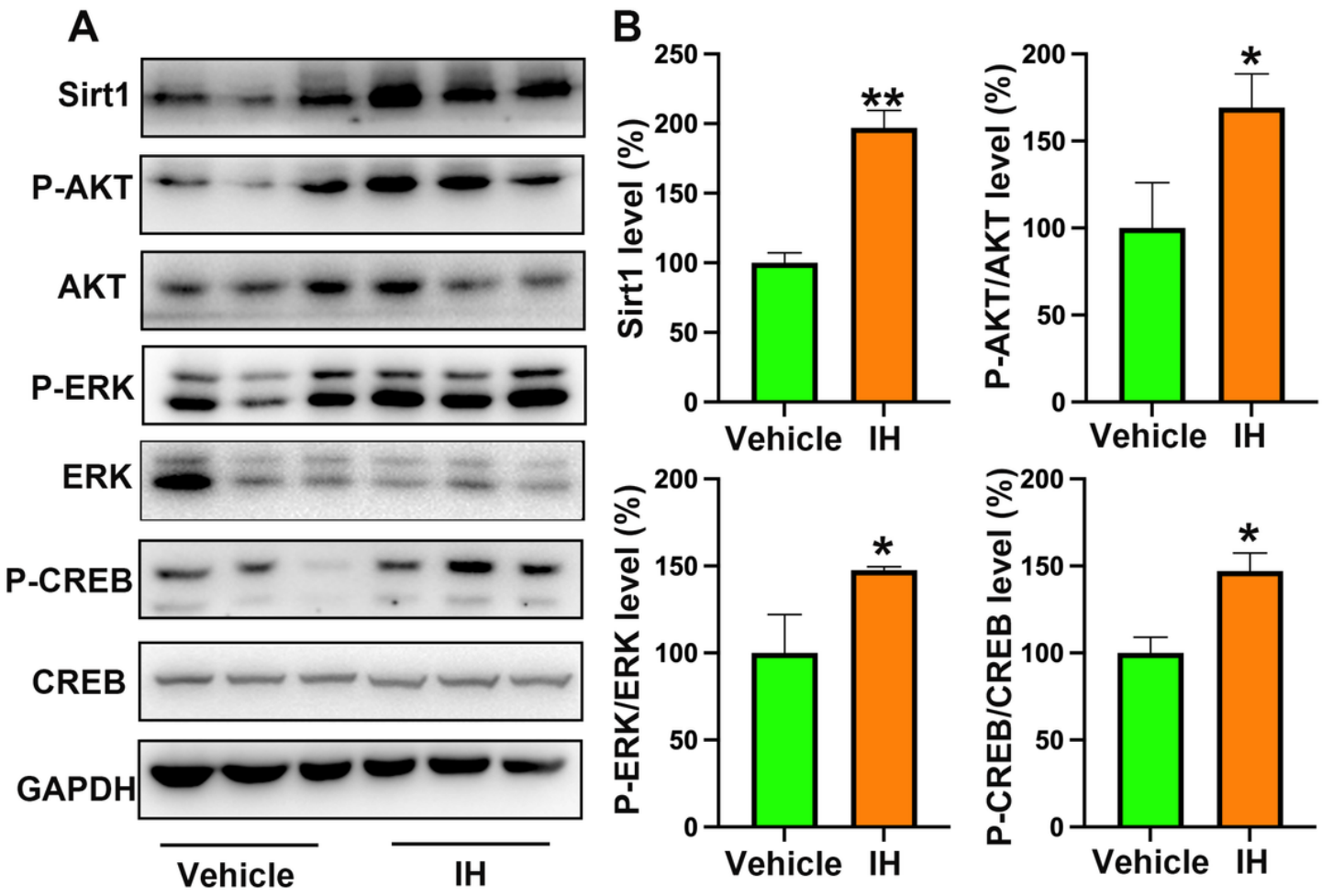



**Figure 6**

**IH activated the Sirt1/AKT/ERK/CREB signaling pathways in the APP/PS1 mice.** Four-month-old APP/PS1 mice were treated with IH for 3 months. (A-B) Immunoblot analysis showed the expression levels of Sirt1, p-AKT, AKT, p-ERK , ERK, p-CREB and CREB. Data were presented as the mean ± SD; n = 6, * $P < 0.05$; * $P < 0.05$; ** $P < 0.01$