CZ2HF mitigates β-amyloid 25-35 fragment-induced learning and memory impairment through inhibition of neuroinflammation and apoptosis in rats

LINGRONG ZENG[1,2], JIANMEI GAO[3], YUANYUAN DENG[1,2], JINGSHAN SHI[1,2] and QIHAI GONG[1,2]

[1]Key Laboratory of Basic Pharmacology of Ministry of Education, Department of Pharmacology, Zunyi Medical University;
[2]Joint International Research Laboratory of Ethnomedicine of Ministry of Education, Zunyi Medical University;
[3]Department of Clinical Pharmacotherapeutics, School of Pharmacy, Zunyi Medical University,
Zunyi, Guizhou 563000, P.R. China

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Abstract. Cu-zhi-2-hao-fang (CZ2HF), a traditional Chinese medicine, has been used clinically for the treatment of amnesia. However, whether CZ2HF is capable of alleviating learning and memory impairment in Alzheimer's disease (AD) remains to be elucidated. The present study was designed to explore the effect and mechanism of CZ2HF on β-amyloid 25-35 ($A\beta_{25-35}$)-induced impairment in the learning and memory of rats. Morris water maze test was used to determine spatial learning and memory ability in $A\beta_{25-35}$-induced AD rats and hippocampal neuronal damage and apoptosis were observed using hematoxylin and eosin staining, Nissl staining and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays, respectively. The levels of β-amyloid 1-42 ($A\beta_{1-42}$), pro-inflammatory factors, such as cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and apoptosis-associated genes including B cell leukemia/lymphoma 2 (Bcl-2), Bcl-2-associated X, apoptosis regulator (Bax), pro-caspase-3, inhibitor of κB (IκB-α) degradation and phosphorylated-nuclear factor-κB p65 (p-NF-κB p65) activation were analyzed using western blotting. The findings of the present study revealed that CZ2HF treatment significantly attenuated $A\beta_{25-35}$-induced cognitive impairments in rats. Subsequently, CZ2HF treatment markedly inhibited neuronal damage and deletions. Furthermore, CZ2HF reduced TNF-α, IL-1β, COX-2 protein expression levels, Bax/Bcl-2 ratio, and reduced $A\beta_{1-42}$ and active-caspase-3 levels. In addition, IκB-α degradation and p-NF-κB p65 activation were reduced by CZ2HF. These findings suggested that CZ2HF treatment improved $A\beta_{25-35}$-induced learning and memory impairment and hippocampal neuronal injury, and its underlying mechanism may be due to the inhibition of neuroinflammation and neuronal apoptosis. CZ2HF may be a potential agent for the treatment of AD.

Correspondence to: Professor Qihai Gong, Key Laboratory of Basic Pharmacology of Ministry of Education, Department of Pharmacology, Zunyi Medical University, 6 Xuefu West Road, Zunyi, Guizhou 563000, P. R. China
E-mail: gqh@zmc.edu.cn

Abbreviations: AD, Alzheimer's disease; CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25-35}$, β-amyloid 25-35; MWM, Morris water maze; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; NF-κB p65, nuclear factor-κB p65

Key words: cu-zhi-2-hao-fang, β-amyloid 25-35, learning, memory, neuroinflammation, apoptosis

Introduction

Alzheimer's disease (AD) is a major type of dementia in the elderly, which is characterized by progressive learning and memory impairment. The present consensus is that the typical pathological features of AD are extracellular β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles in the brain, accompanied by neuronal damage or loss (1,2). Due to an increase in the aging population in recent years, there are currently ~46 million dementia patients worldwide and the number of patients enrolled in 2,050 is expected to increase to 135 million, which will put a great economic burden on society and patients' families (3). The pathogenesis of AD is complex, and remains to be fully elucidated. It has been previously established that the Aβ cascade theory has an important role in the development of AD (4). Aβ is derived from β-secretase and γ-secretase and the deposition of Aβ, particularly $A\beta_{1-42}$ leads to neurotoxicity and neurodegeneration during the progress of AD (5). Physiologically, the formation and removal of Aβ in the brain is maintained in a dynamic equilibrium state; however, when this balance is dysregulated, it may lead to abnormal deposition of Aβ and disturb the physiological activity of the neuronal cells (6). A previous study showed that Aβ deposition produced a sequence of cascade reactions, such as exacerbation of the inflammatory response, including increased interleukin-1β (IL-1β), tumor necrosis factor

(TNF-α), cyclooxygenase-2 (COX-2) expression and levels of nuclear factor-κB (NF-κB) (7). Additionally, these inflammatory factors may increase β-amyloid precursor protein (APP) expression in the brain and upregulate the activity of γ-secretase; therefore, the levels of Aβ were increased in turn (8). In addition, a previous study indicated that Aβ deposition in the brain may induce neuronal apoptosis, which may lead to further learning and memory impairment. B-cell lymphoma-2 (Bcl-2) gene family members, such as Bcl-2 and Bcl-2-associated X, apoptosis regulator (Bax), have important roles in the process of apoptosis (9). It is of note that it has been previously demonstrated that the learning and memory impairment of the $A\beta_{1\text{-}40}$-induced AD rats may be ameliorated through regulation of the apoptosis-associated genes, such as Bax and Bcl-2. Therefore, inhibition of neuroinflammation and neuronal apoptosis may be used as a promising strategy for the clinical treatment of AD.

Evidence from clinical and experimental trials revealed that cholinesterase inhibitors such as donepezil, tacrine and galantamine, and a N-methyl-D-aspartate receptor antagonists, such as memantine may be used as AD-treatment agents (10-12). However, these drugs had limited use in a clinical setting due to their single target, their price and multiple side effects (13). Therefore, Traditional Chinese Medicine (TCM) may be a promising treatment method for AD as it has advantages of multi-targets and reduced side effects.

The cu-zhi-2-hao-fang (CZ2HF) decoction, an empirical formula of TCM, which consisted of *Herba Epimedium*, *Rhizoma curculiginis*, *Morinda officinalis*, *Acorus gramineus*, *Lycium barbarum*, *Scrophularia ningpoensis*, *Cinnamomum cassia* Presl, *Rhizoma zingiberis* (Table I). According to TCM theory, *Herba Epimedium* is the primary component in this decotion, which is one of the traditional Chinese herbs for treating various diseases, such as AD and its main active ingredients, icariin and icariside II downregulate $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ expression levels in the brain of Tg2576 transgenic mice to mitigate learning and memory impairment and reduce TNF-α, COX-2, IL-1β expression levels and neuronal apoptosis to improve $A\beta_{25\text{-}35}$-induced learning and memory impairments in rats (14-16). CZ2HF was used to clinically prevent and treat amnesia. However, whether CZ2HF may alleviate $A\beta_{25\text{-}35}$-induced learning and memory impairment and its underlying mechanism remains to be elucidated. Therefore, the present study was designed to investigate the effect of CZ2HF on $A\beta_{25\text{-}35}$-induced learning and memory impairment and further elucidate its possible action mechanism.

Materials and methods

Agents. Donepezil hydrochloride (1511010) was obtained from Affiliated Hospital of Zunyi Medical University (Zunyi, China), $A\beta_{25\text{-}35}$ (the amino acid sequence is Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met) was purchased from Sigma-Aldrich (cat. no. A4559; Merck Millipore, Darmstadt, Germany), primary antibodies of COX-2 (cat. no. ab15191), active-caspase-3 (cat. no. ab13847), Bax (cat. no. ab7977), Bcl-2 (cat. no. ab7973), IL-1β (cat. no. ab9787), TNF-α (cat. no. ab66579), $A\beta_{1\text{-}42}$ (cat. no. ab10148) were acquired from Abcam (Cambridge, UK), pro-caspase-3 antibody (cat. no. sc-7148) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), primary antibodies of inhibitor of κB (IκB-α; cat. no. 9242), phosphorylated (p)-nuclear factor (NF)-κB p65 (cat. no. 3033), NF-κB p65 (cat. no. 8242) were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Preparation of CZ2HF decoction. CZ2HF consisted of 8 ingredients, as presented in Table I. CZ2HF was provided by Affiliated Hospital of Zunyi Medical University and identified by Professor Jianwen Yang (School of Pharmacy, Zunyi Medical University, Zunyi, China). Briefly, a mixture of 9 g *Herba Epimedium*, 9 g *Rhizoma curculiginis*, 9 g Morinda officinalis, 9 g Acorus calamus, 9 g *Lycium barbarum*, 9 g *Scrophularia ningpoensis*, 5 g *Cinnamomum cassia* Presl, 5 g Zingiberis rhizome was soaked for 60 min with 1,000 ml distilled water and boiled for 1.5 h. Subsequently, the filtrate was gathered and the residue was boiled again for an additional 1 h with distilled water. The extraction was further condensed, combined and lyophilized according to the protocol as previously described (17). The yield was 21.6% relative to the original crude quantity.

Preparation of animal model and drug treatment. A total of 98 healthy male Sprague-Dawley (SD) rats (250-300 g) were purchased from the Laboratory Animal Center of the Third Military Medical University (Chongqing, China; certificate no. SCXK2012-0011). The rats were housed in specific pathogen-free conditions, under a 12-h light/dark cycle, temperature was 22±1˚C, humidity was 60±2% and were given free access to food and water. All experiments were approved by the Ethics Committee and performed according to the current guide for the care and use of laboratory animal standard, which was set up by Zunyi Medical University Animal Studies Committee (argument number [2015] 2-043). The rats were randomly divided into 7 groups as follows: i) Sham; ii) sham+CZ2HF (400 mg/kg); iii) model ($A\beta_{25\text{-}35}$); iv) $A\beta_{25\text{-}35}$+CZ2HF (100 mg/kg); v) $A\beta_{25\text{-}35}$+CZ2HF (200 mg/kg); vi) $A\beta_{25\text{-}35}$+CZ2HF (400 mg/kg); and vii) $A\beta_{25\text{-}35}$+donepezil (1.0 mg/kg) as the positive drug group (n=14 rats per group. Briefly, $A\beta_{25\text{-}35}$ (1 mg) was dissolved in 500 μl saline, configured as 2.0 μg/μl solution, placed in 37˚C incubator for 4 days, in order to induce a clustered state to enhance its toxicity (18). Subsequently, SD rats were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium, the rat's brain was fixed a stereotaxic device, and the following hippocampus needle coordinates were used: 3.5 mm posterior to the bregma, 2.5 mm lateral to the sagittal suture, 3.5 mm beneath the surface of brain. The needle was retained in the bilateral hippocampi for 5 min and the 5 μl $A\beta_{25\text{-}35}$ was injected. Various doses of CZ2HF were administered orally daily for a continuous period for 15 days. The rats in CZ2HF group were treated with CZ2HF alone, and the sham group were given double-distilled water at an equal volume to the CZ2HF solution.

Morris water maze test. The Morris water maze test (MWM) was performed in order to determine the learning and memory function of the rats from the 7 groups as described in our previous study (19). The rats were trained and exposed to 4 successive memory acquisition trials in the MWM to analyze their capacity to escape and find the platform, which was performed daily between days 11 and 16 after the

Table I. Composition of cu-zhi-2-hao-fang.

Common name	Latin name	Quantity (g)
Herba Epimedium	*Epimedium brevicornu* Maxim.	9
Rhizoma curculiginis	*Curculigo orchioides* Gaertn.	9
Morinda officinalis	*Morinda officinalis* How.	9
Acorus gramineus	*Acorus tatarinowii*.	9
Lycium barbarum	*Lycium barbarum* L.	9
Scrophularia ningpoensis	*Scrophularia ningpoensis* Hemsl.	9
Cinnamomum cassia Presl	*Cassia Twig*	5
Rhizoma zingiberis	*Zingiber officinale* Roscoe	5

$A\beta_{25-35}$ injection. On day 16, the spatial probe experiment was performed to detect the ability of spatial memory. All SD rats were subjected to anesthesia by 0.3 ml of 2% pentobarbital sodium injection after intraperitoneal examination of the MWM.

Hematoxylin and eosin (H&E) staining. Following fixation in 4% for 48 h (pH 7.4), the brains were removed, fixed with 4% paraformaldehyde at 4˚C, dehydrated and embedded in paraffin. Subsequently, 3-μm thick frozen sections were prepared and H&E staining at room temperature for 12 min was used to detect pathological changes in the CA1 region of hippocampal tissue by an independent pathologist. Images of the histopathological examination were observed using a light microscope. Three rats per group were used for H&E staining.

Nissl staining. Brains were fixed with 4% paraformaldehyde at 4˚C for 48 h and subsequently embedded in paraffin. Sections (3-μm thick) of rat brain tissue were stained with toluidine blue at 60˚C for 10 min. The Nissl bodies were stained blue-purple in the CA1 region of the hippocampus to estimate the morphological changes of neurons in the CA1 area and assessed by a light microscope.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining. Apoptosis was evaluated using TUNEL staining with the In Situ Cell Death Detection kit (cat. no. 11684817910; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. The brain slices of rats from each group were washed twice with double distilled water for 5 min, then the sections were soaked in 3% H_2O_2 solution for 15 min. Subsequently, the slices were washed with PBS 3 times for 5 min, placed in a dark chamber and proteinase K was added to the working solution. Next, the brain slices were incubated for 60 min at 37 ˚C with TUNEL reaction mixture and the sections were washed with PBS again and incubated for 30 min at 37˚C with converter-POD work liquid. The sections were subsequently treated with DAB substrate solution and washed again with PBS. A total of 3 images were captured randomly for each section and counted using a fluorescent microscope as described in our previous study (19).

Western blotting. Expression levels of TNF-α, IL-1β, COX-2, IκB-α, NF-κB p65, p-NF-κB p65, Bax, Bcl-2, caspase-3, $A\beta_{1-42}$ were dretermined using western blotting. Briefly, three rats were randomly selected from each group and sacrificed, the hippocampal tissues were dissected and immediately frozen at -80˚C. Then, the subsequent procedures were performed as described in our previous study (20). The corresponding proteins in this study were analyzed using primary antibodies against TNF-α (1:2,000), IL-1β (1:1,000), COX (1:1,000), IκB-α (1:2,000), NF-κB p65 (1:1,000), p-NF-κB p65 (1:1,000); Bax (1:500), Bcl-2 (1: 500), pro-caspase-3 (1:1,000), active-caspase-3 (1:1,000) and $A\beta_{1-42}$ (1:2,000). The membranes were incubated overnight with the primary antibodies at 4˚C. Susbequently, the membranes were washed twice with TBST and incubated with secondary antibodies goat anti-rabbit IgG H&L (cat. no. ab6702; 1:1,000; Abcam) for 2 h at room temperature. The blots were visualized using Davinch-Chemi™ imaging system and the relative band optical intensity was quantified using Quantity One 1-D analysis software version 4.52 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were expressed as the mean ± standard error of the mean and analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance and differences among means were analyzed using Dunnett's test or Tukey-Kramer's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CZ2HF mitigates $A\beta_{25-35}$-induced learning and memory impairment in rats. In order to investigate whether CZ2HF may alleviate the learning and memory impairment induced by $A\beta_{25-35}$ in rats, the spatial learning and memory function of rats was determined using the MWM test, which was performed from day 11 to day 16 after the $A\beta_{25-35}$ injection (Fig. 1A). The findings revealed that the model group rats had a notably elevated escape latency compared with the sham group, indicating that injection of $A\beta_{25-35}$ impaired their ability of spatial learning. However, CZ2HF attenuated escape latency compared with model group ($F_{6,85}=2.217$; $P< 0.05$; Fig. 1B). Following the hidden platform training on day 16, a spatial probe test was performed to determine spatial memory abilities by counting the time spent in the target quadrant of the rats of various groups (14). The findings revealed that the rats in the model group spent shorter time in the target

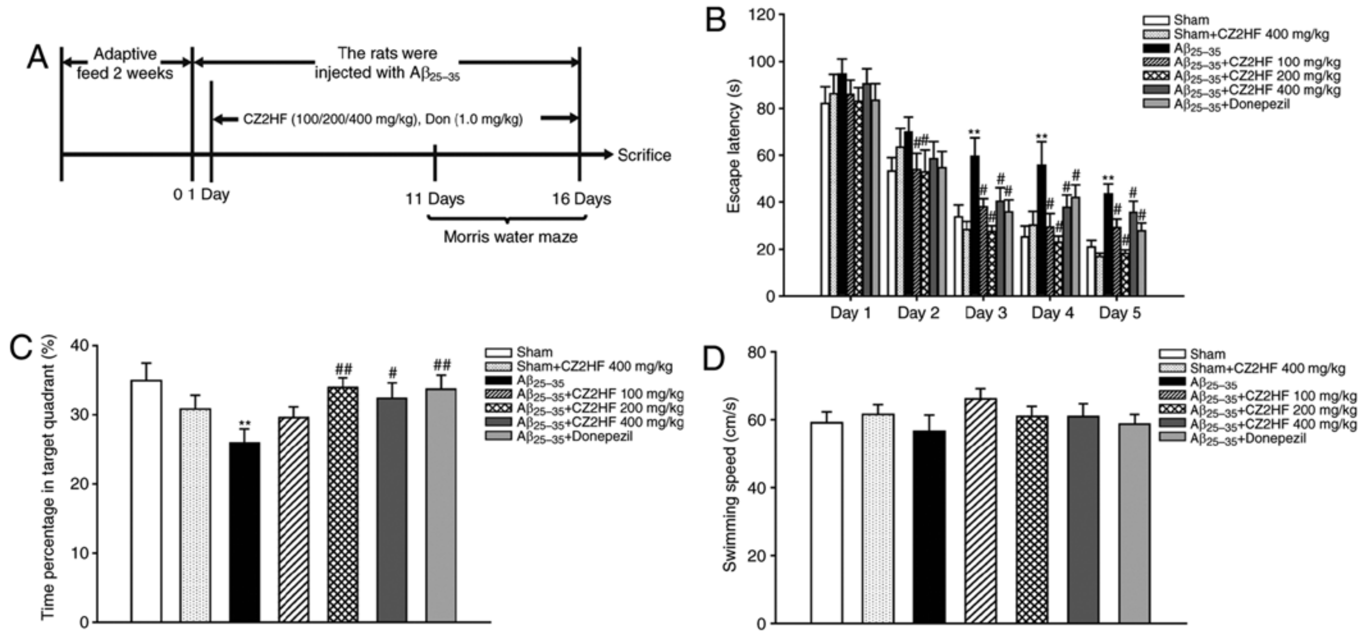

Figure 1. Effect of CZ2HF on $A\beta_{25\text{-}35}$-induced learning and memory impairments in rats. (A) Experimental design. (B) Escape latency. (C) Time spent in target quadrant. (D) Swimming speed. **P<0.01 vs. sham; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the $A\beta_{25\text{-}35}$. Sham (n=14), $A\beta_{25\text{-}35}$ (n=11), Sham+CZ2HF (400 mg/kg) (n=13), $A\beta_{25\text{-}35}$+CZ2HF (100 mg/kg) (n=12), $A\beta_{25\text{-}35}$+CZ2HF (200 mg/kg) (n=12), $A\beta_{25\text{-}35}$+CZ2HF (400 mg/kg) (n=13), $A\beta_{25\text{-}35}$+Don (1.0 mg/kg) (n=13). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35; Don, donepezil.

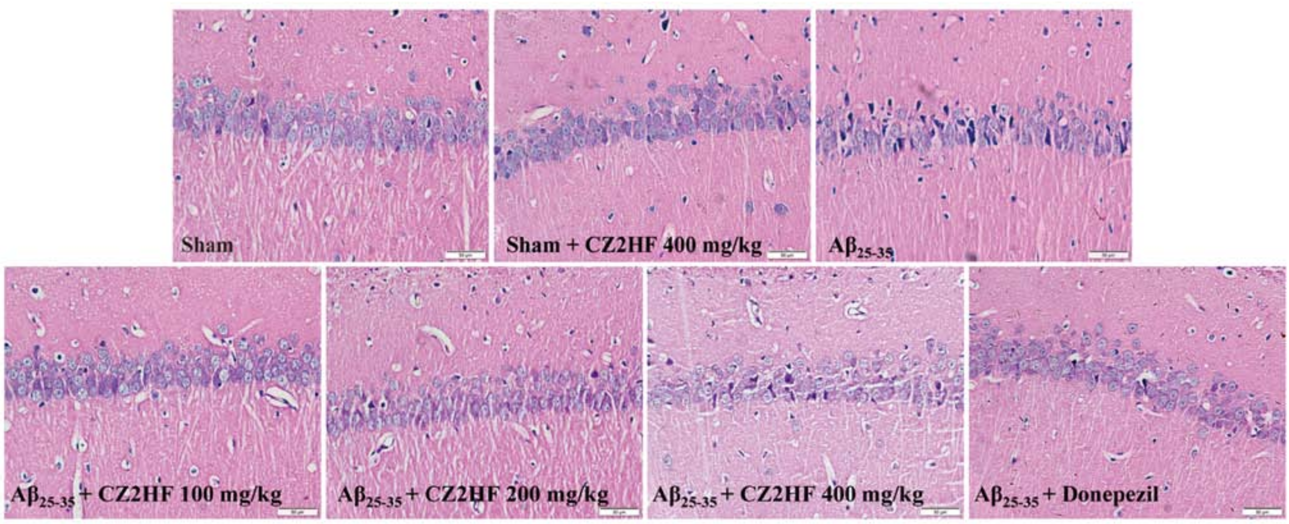

Figure 2. Effect of CZ2HF on hippocampal neuronal morphological alterations induced by $A\beta_{25\text{-}35}$ in rats. Scale bar, 50 μm. CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35.

quadrant compared with the sham group rats. However, CZ2HF increased the retention time in the target quadrant compared with the model group (Fig. 1C). No significant difference was identified between the swimming speed of the different treatment rat groups (Fig. 1D), indicating that CZ2HF and donepezil did not affect the motor function of rats.

CZ2HF reduces $A\beta_{25\text{-}35}$-induced neuronal injury of hippocampus. H&E and Nissl staining were used to evaluate the effects of CZ2HF on the morphology of hippocampal neurons and neuronal injury. The current findings revealed that the neurons in the CA1 region of the hippocampus of rats in the sham group had high density, the nuclei and cytoplasm were homogeneous and the edges were clear. However, in the model group, the neurons were disordered, their density was low and a large number of cells were nucleated, indicating that $A\beta_{25\text{-}35}$ damaged the neuronal cells in the rat hippocampus. However, CZ2HF (100, 200, 400 mg/kg) and donepezil treatment notably ameliorated the neuronal structure and density (Fig. 2). Furthermore, the results of Nissl staining revealed that the neurons in CA1 region of sham were arranged neatly and densely. Additionally, the nuclei and cytoplasm were stained uniformly and the structure of neuron was clear and complete (Fig. 3A). However, the cellular structure became unclear, the cell density was lower and the neurons were disordered in the model group, indicating that $A\beta_{25\text{-}35}$ damaged the hippocampal

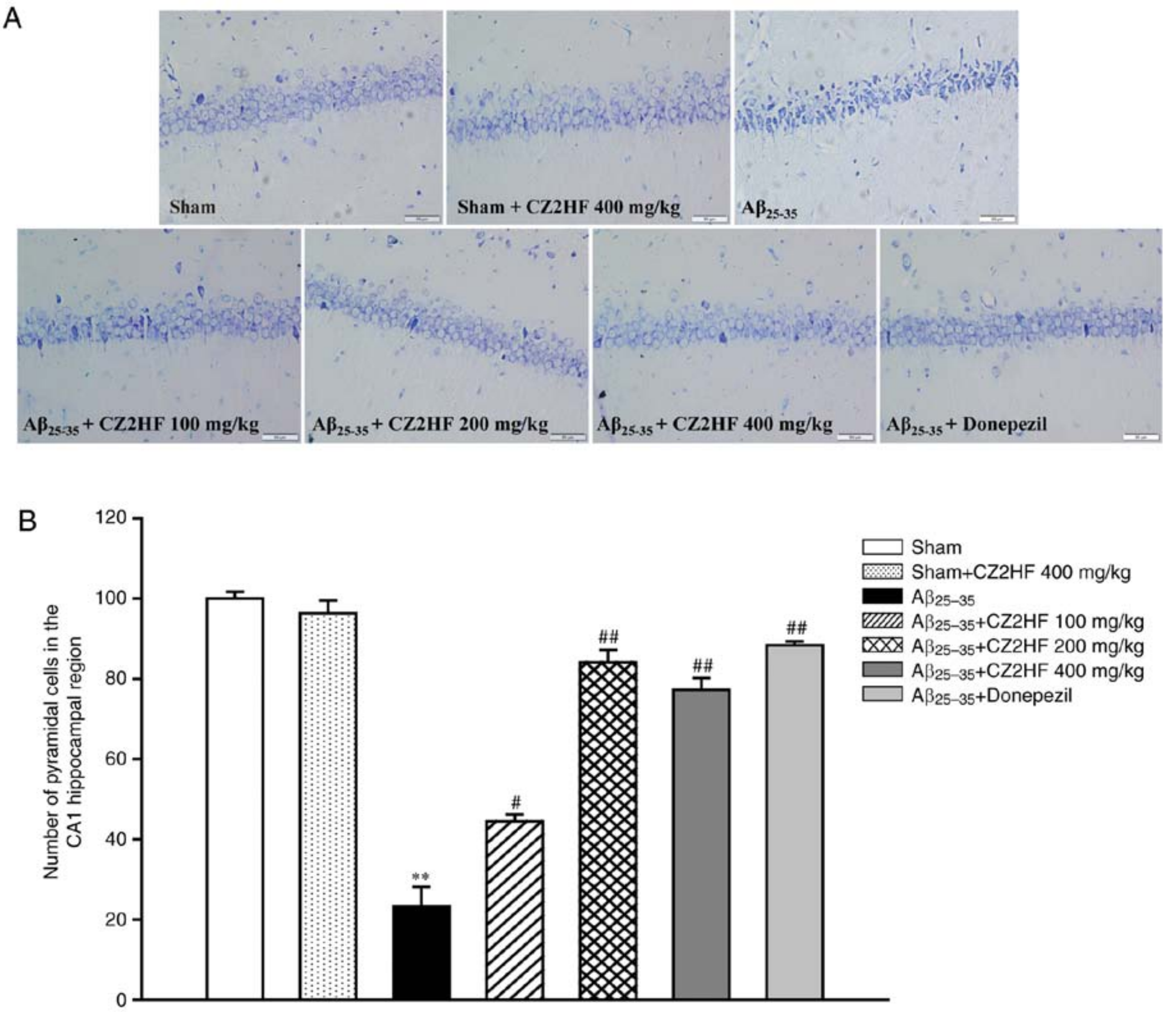

Figure 3. Effect of CZ2HF on hippocampal neuronal injury induced by $A\beta_{25-35}$ in rats. (A) Representative images of Nissl staining. Scale bar, 50 μm. (B) Number of pyramidal cells in the CA1 hippocampal region. $^{**}P<0.01$ vs. sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the $A\beta_{25-35}$ (n=4). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25-35}$, β-amyloid 25-35.

neurons. CZ2HF treatment ameliorated the $A\beta_{25-35}$-induced the injury of neuronal structure (Fig. 3A). Meanwhile, these effects were also confirmed by the number of pyramidal cells counted in the CA1 hippocampal region of the rats (Fig. 3B).

CZ2HF attenuates the level of hippocampal $A\beta_{1-42}$ induced by $A\beta_{25-35}$. Western blotting was used to detect the level of $A\beta_{1-42}$ in the rat hippocampus induced by $A\beta_{25-35}$ exposure (Fig. 4A). The present study determined that the level of $A\beta_{1-42}$ was significantly increased in model group; however, CZ2HF (100, 200, 400 mg/kg) and donepezil notably reduced the level of $A\beta_{1-42}$ in the rat hippocampus injected with $A\beta_{25-35}$, suggesting that CZ2HF and donepezil may block $A\beta_{25-35}$-induced $A\beta_{1-42}$ increase ($F_{6,14}$=6.283; $P<0.01$; Fig. 4B).

CZ2HF represses $A\beta_{25-35}$ -induced neuroinflammatory factors in rats. In order to determine the role of neuroinflammation during the process of $A\beta_{25-35}$-induced cognitive impairment in rats, the inflammatory factors in hippocampi were determined using western blotting (Fig. 5A). It was shown that TNF-α, IL-1β, and COX2 expression levels were increased in the model group, suggesting that $A\beta_{25-35}$ triggered the inflammatory response. However, CZ2HF (200, 400 mg/kg) and

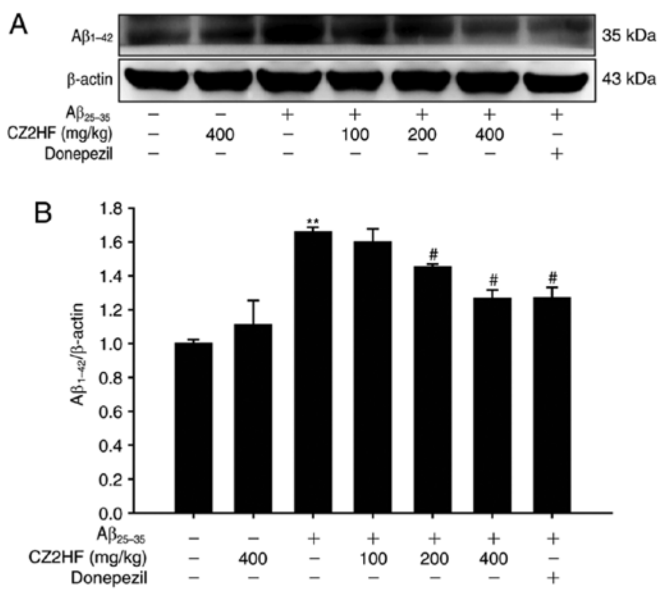

Figure 4. Effect of CZ2HF on the levels of $A\beta_{1-42}$ in hippocampus induced by $A\beta_{25-35}$ in rats. (A) Representative western blot analysis of $A\beta_{1-42}$. (B) Quantification of $A\beta_{1-42}$ protein expression levels. $^{**}P<0.01$ vs. sham; $^{\#}P<0.05$ vs. the $A\beta_{25-35}$ (n=3). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{1-42}$, β-amyloid 1-42; $A\beta_{25-35}$, β-amyloid 25-35.

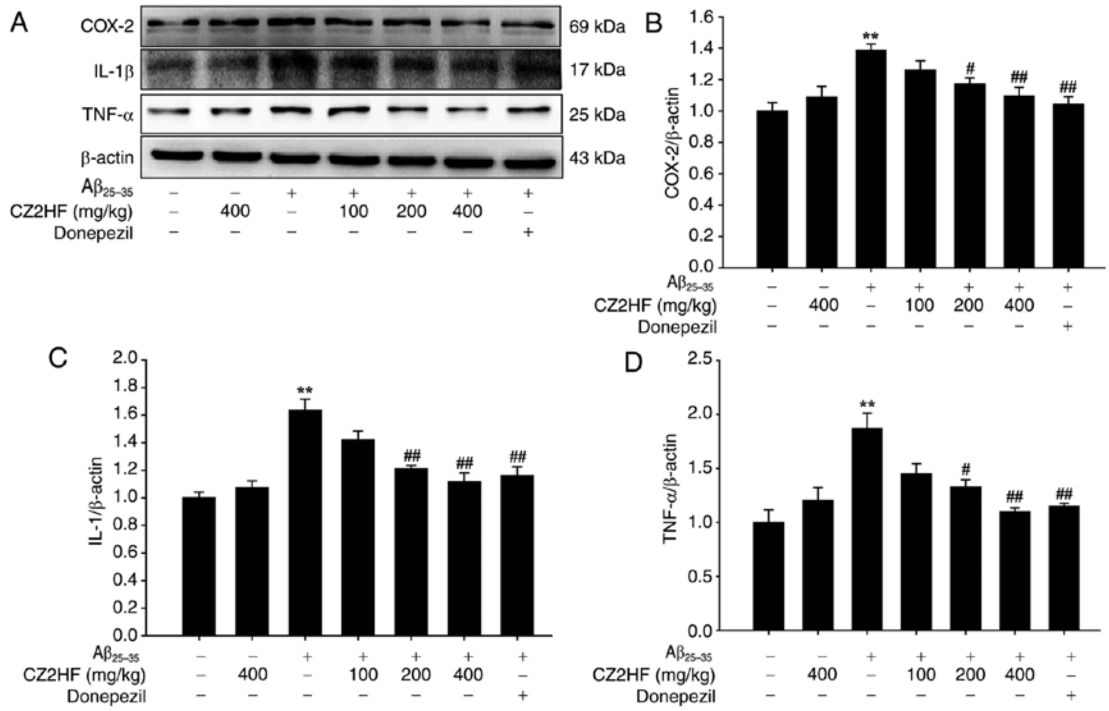

Figure 5. Effect of CZ2HF on COX-2, TNF-α and IL-1β expression levels in hippocampus induced by $A\beta_{25\text{-}35}$ in rats. (A) Representative images of western blotting for COX-2, TNF-α, IL-1β protein expression. Quantification of (B) COX-2, (C) IL-1β and (D) TNF-α protein expression levels. $^{**}P<0.01$ vs. sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. $A\beta_{25\text{-}35}$ (n=3). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35; COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

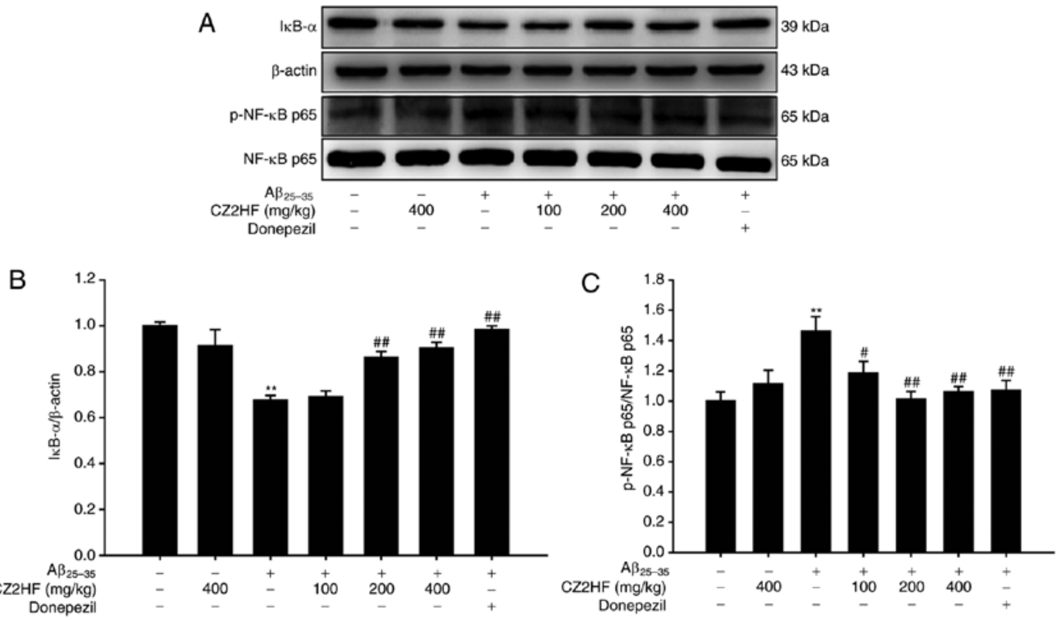

Figure 6. Effect of CZ2HF on the expression levels of IκB-α and p-NF-κB p65 in hippocampus induced by $A\beta_{25\text{-}35}$ in rats. (A) Representative images of western blotting for IκB-α and p-NF-κB p65 levels. Quantification of (B) IκB-α and (C) p-NF-κB p65 levels. $^{**}P<0.01$ vs. the sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the $A\beta_{25\text{-}35}$ (n=3). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35; p-, phosphorylated; NF-κB p65, nuclear factor-κB p65; IκB-α, inhibitor of κB.

donepezil treatment significantly reduced the increase in these inflammatory factors including COX2 ($F_{6,14}$=2.96; P<0.05; Fig. 5B), IL-1β ($F_{6,14}$=7.802; P<0.01; Fig. 5C) and TNF-α ($F_{6,14}$=6.082; P<0.01; Fig. 5D). Furthermore, as NF-κB has a key role in the development of AD (Fig. 6A) and it is upstream of the aforementioned inflammatory factors, these findings were confirmed that CZ2HF significantly increased the level of IκB-α ($F_{6,14}$=13.850; P<0.05; Fig. 6B) and reduced the p-NF-κB p65 level ($F_{6,14}$=3.829; P<0.05; Fig. 6C). Therefore, CZ2HF may block the phosphorylation of NF-κB p65 induced by $A\beta_{25\text{-}35}$.

CZ2HF inhibits $A\beta_{25\text{-}35}$-induced hippocampal neuronal apoptosis in rats. The effect of CZ2HF on the apoptosis of

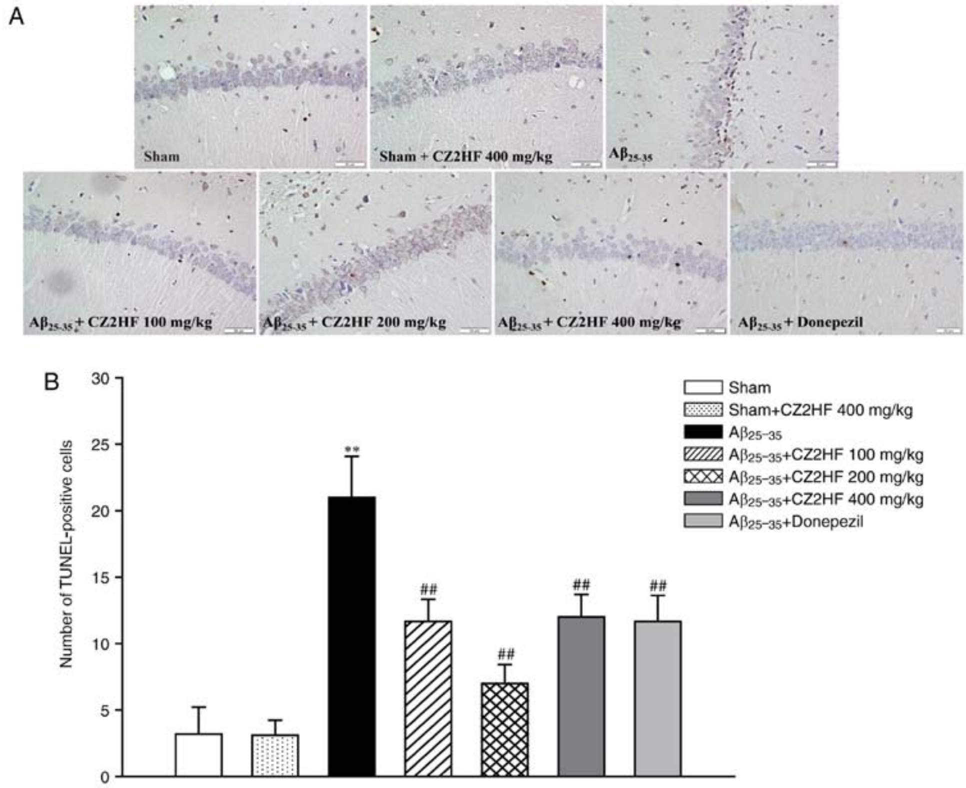

Figure 7. Effect of CZ2HF on $A\beta_{25-35}$-induced hippocampal neuronal apoptosis in rats. (A) Representative images of TUNEL staining. Scale bar, 50 μm. (B) Number of TUNEL-positive cells. $^{**}P<0.01$ vs. sham; $^{\#\#}P<0.01$ vs. $A\beta_{25-35}$ (n=4). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25-35}$, β-amyloid 25-35; $A\beta_{1-42}$, β-amyloid 1-42; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

hippocampal neurons exposed to $A\beta_{25-35}$ was evaluated using TUNEL staining (Fig. 7A). The findings demonstrated that the number of apoptotic cells in the model group was significantly increased compared with the sham group. However, the different doses of CZ2HF markedly attenuated the number of apoptotic cells (Fig. 7B).

CZ2HF suppresses the increase in Bax and caspase-3 and the decrease in Bcl-2 expression levels. To further investigate the possible effects of CZ2HF on apoptosis-associated proteins in $A\beta_{25-35}$-induced hippocampal neuronal apoptosis, the Bax and Bcl-2 protein levels, and active-caspase-3 level were determined by western blotting (Fig. 8). The present study determined that CZ2HF (100, 200 and 400 mg/kg) downregulated Bax expression and upregulated Bcl-2 expression; therefore, the ratio of Bax/Bcl-2 was reduced, which reversed the $A\beta_{25-35}$-induced increase in the Bax/Bcl-2 ratio ($F_{6,14}=2.955$, $P<0.05$; $F_{6,14}=3.063$, $P<0.05$). Additionally, CZ2HF (200, 400 mg/kg) also reduced the level of active-caspase-3 and limited the decrease of pro-caspase-3 compared with the model group ($F_{6,14}=7.867$; $P<0.01$). These findings suggested that CZ2HF may reduce $A\beta_{25-35}$-induced hippocampal neuronal apoptosis by regulating apoptosis-associated proteins (Fig. 8).

Discussion

The findings in the current study suggested that CZ2HF may be a promising agent for the treatment of AD. CZ2HF significantly attenuated $A\beta_{25-35}$-induced cognitive impairments and inhibited neuronal damage and deletions in rats. Additionally, CZ2HF reduced the protein expression of TNF-α, IL-1β, COX-2, the Bax/Bcl-2 ratio, and reduced the levels of $A\beta_{1-42}$ and active-caspase-3. Furthermore, IκB-α degradation and p-NF-κB p65 activation was repressed by CZ2HF.

Accumulating evidence demonstrated that $A\beta_{25-35}$ is the key fragment of full-length $A\beta_{1-42}$ and the acute injection of $A\beta_{25-35}$ into rat cerebral ventricle may lead to neurotoxic effects similar to those produced by the $A\beta_{1-40}$; however, the presence of the $A\beta_{25-35}$ fragment in the AD brains remains to be determined (21,22). Previous studies indicated that bilateral hippocampal injection of $A\beta_{25-35}$ may induce an AD learning and memory impairment model in rats, which has been widely used in AD research (23,24). Therefore, $A\beta_{25-35}$-induced AD rat model was used to investigate the effects and mechanism of CZ2HF on learning and memory impairment in the current study using methodology described in our previous study (14). Additionally, a MWM test was performed to identify the primary effect of CZ2HF on $A\beta_{25-35}$-induced learning and memory function injury in the rats. As $A\beta_{25-35}$-induced learning and memory function exhibits self-limitation, Contextual and Cued Fear Conditioning Test, which may confirm the association between hippocampal dependence and learning, and memory function will be performed in future studies. In addition, as the present study was preliminary in order to determine the effect of CZ2HF on $A\beta_{25-35}$-induced AD; therefore, 3 or 4 rats were used from each group in the present study. Therefore, the number of rats used for each molecular or histological experiment was 3 or 4. The current findings revealed that CZ2HF significantly ameliorated learning and memory dysfunction in AD rats using the MWM test.

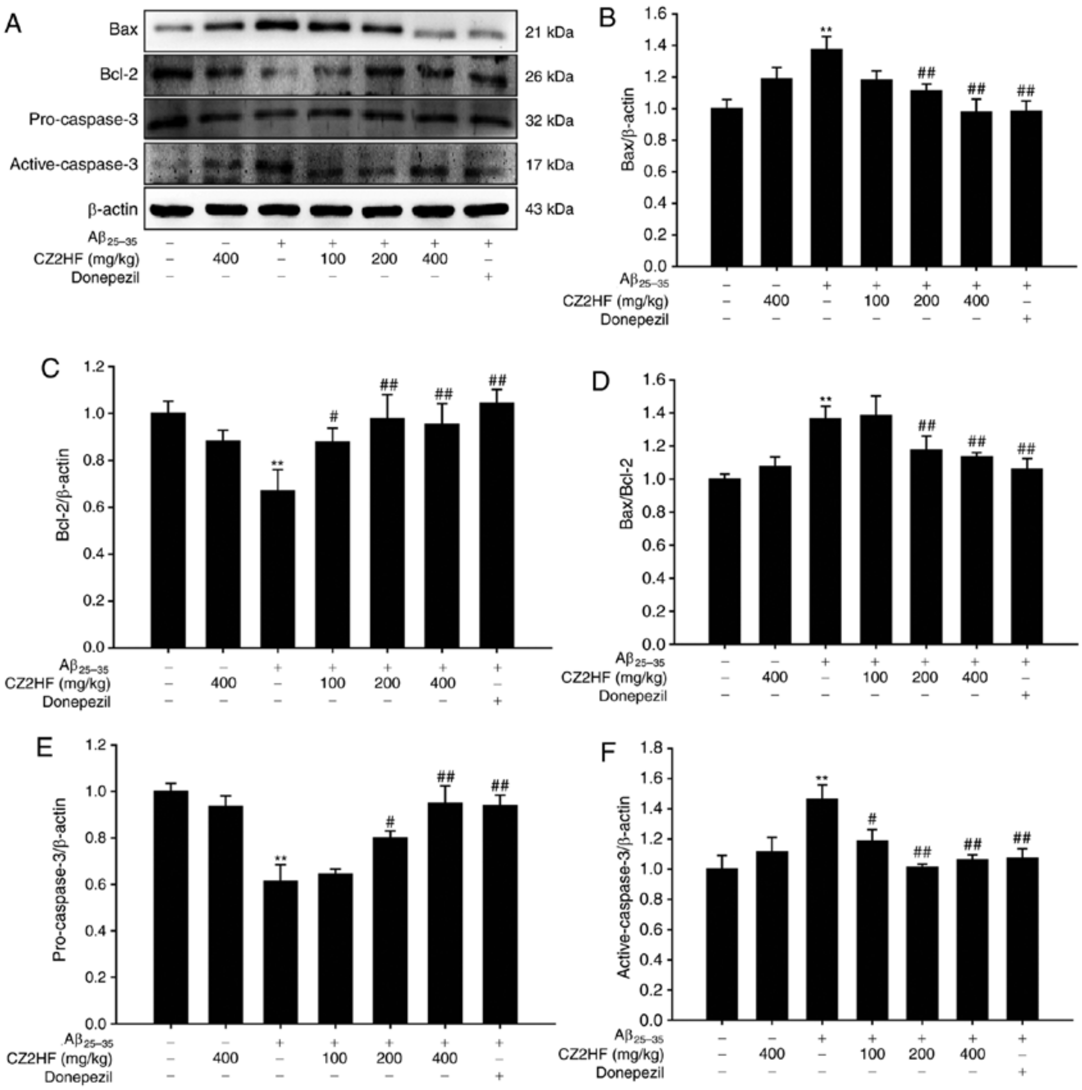

Figure 8. Effect of CZ2HF on the protein expression of Bax, Bcl-2 and caspase-3 induced by $A\beta_{25\text{-}35}$ in rats. (A) Representative western blotting images of Bax, Bcl-2, pro-caspase-3 and active-caspase-3. Quantification of (B) Bax, (C) Bcl-2, (D) Bax/Bcl-2 ratio, (E) pro-caspase-3 and (F) active-caspase-3. $^{**}P<0.01$ vs. sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. $A\beta_{25\text{-}35}$ (n=3). CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35; Bax, Bcl-2-associated X, apoptosis regulator; Bcl-2, B cell leukemia/lymphoma 2.

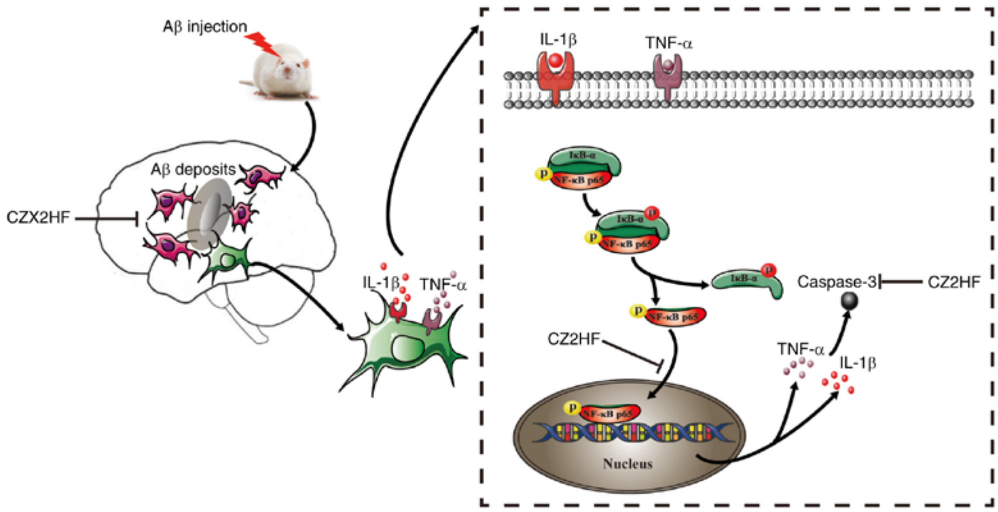

Figure 9. Schematic representation of the effect and underlying mechanism of CZ2HF on $A\beta_{25\text{-}35}$ induced the learning and memory impairment in rats. The crosstalk between neuroinflammation and apoptosis signaling pathway is involved in the inhibitory effect of CZ2HF on $A\beta_{25\text{-}35}$-induced hippocampal neuronal damage. CZ2HF, cu-zhi-2-hao-fang; $A\beta_{25\text{-}35}$, β-amyloid 25-35; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IκB-α, inhibitor of κB.

Furthermore, CZ2HF inhibited the $A\beta_{25-35}$-induced reduction of the number of neurons, which was observed by the H&E and Nissl staining. This indicated that CZ2HF had beneficial effects on learning and memory impairment, attenuation of hippocampal neuronal damage or loss. Nevertheless, the underlying mechanism of CZ2HF must be further elucidated.

It has been previously established that Aβ is derived from the ordered hydrolysis of APP and Aβ fragments of 39-43 amino acids were formed in this process, including $A\beta_{1-40}$ and $A\beta_{1-42}$ (25). The present study showed that $A\beta_{1-42}$ with highest toxicity were significantly increased in $A\beta_{25-35}$-induced learning and memory impairment in the rat brains, which was consistent with the report that $A\beta_{25-35}$-induced learning and memory impairment was accompanied with a greater $A\beta_{1-42}$ protein level (26). Previous studies indicated that the inflammatory factors, including COX-2, IL-1 and TNF-α may be agglutinated by upregulating $A\beta_{1-42}$ in the central nervous system and subsequently lead to a learning and memory disorder (27). The findings in the present study demonstrated that $A\beta_{1-42}$ and COX-2, IL-1β and TNF-α levels were increased in $A\beta_{25-35}$-induced AD rats, which was consistent with the fact that the inflammatory cytokines increase the activity of β-secretase and the content of APP, leading to increased $A\beta_{1-42}$ levels, which creates a positive feedback effect aggravates cognitive dysfunction (28). However, CZ2HF and donepezil significantly downregulated TNF-α, IL-1β and COX-2 protein expression, indicating that CZ2HF reduced the decrease in $A\beta_{25-35}$-induced learning and memory impairment both through reducing $A\beta_{1-42}$ level and the inflammatory factors, such as COX-2, IL-1β and TNF-α. Additionally, NF-κB, a vital nuclear transcription factor, is located in the cytoplasm and binds to IκB (29).When the cells are stimulated, IκB is phosphorylated and degraded, which activates NF-κB p65, and upregulates inflammatory factors during the inflammatory process. Additionally, NF-κB is also identified to be upstream of the inflammatory factors (30). The present findings revealed that degradation of IκB-α and the subsequent activation of NF-κB p65 were increased by the $A\beta_{25-35}$ injection treatment. However, CZ2HF significantly reduced the degradation of IκB-α and inhibited NF-κB p65 phosphorylation in $A\beta_{25-35}$-induced AD rats. Therefore, it is possible that CZ2HF ameliorated the learning and memory impairment, at least partly, through regulation of the NF-κB signaling pathway.

Previous studies have indicated that the NF-κB pathway may induce an inflammatory response to release inflammatory factors and lead to neuronal apoptosis, which also contributes to the development and progression of AD (31,32). Bcl-2 family proteins such as the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax have a key role in the process of neuronal apoptosis. Additionally, caspase-3 is the key terminal cleavage enzyme during apoptosis and also executes apoptosis, thereby leading to neuronal cell death (33). The present findings revealed that Bcl-2 was reduced, whereas Bax and caspase-3 were increased in $A\beta_{25-35}$-induced AD rats, which was consistent with a previous report which stated that $A\beta_{25-35}$ may increase the ratio of Bax/Bcl-2 and activate caspase-3, inducing neuronal cell apoptosis (34). However, CZ2HF reversed the aforementioned effects, which confirmed that the beneficial effects of CZ2HF on learning and memory impairment may be associated with inhibition of neuronal cell apoptosis. Additionally, the present findings also indicated that inflammatory response and neuronal apoptosis have an imperative role in the progression of AD and there is a connection between Aβ deposition, neuroinflammation and apoptosis. However, since aqueous extract of TCM contained a large number of polysaccharides, CZ2HF did not exhibit a dose-dependent effect for the treatment of $A\beta_{25-35}$-induced symptoms in AD-like rats. It is of note that on the protein level, CZ2HF exerted beneficial effects in a dose-dependent manner, which may be associated with a potential indirect effect; therefore, compared with other doses, CZ2HF at a dose of 400 mg/kg promoted the apoptosis. The in-depth mechanism of CZ2HF on learning and memory impairment requires further investigation. Additionally, considering that the components of CZ2HF were complex, the mechanism which allows CZ2HF or its exact components to pass though the blood-brain barrier should be investigated in future studies.

In conclusion, the present study demonstrated that CZ2HF ameliorates $A\beta_{25-35}$-induced learning and memory impairment in rats and inhibits the damage of hippocampal neurons. To the best of our knowledge the present study was the first to determine the underlying mechanisms, which may be attributed, at least in part, to repressing the inflammatory response and apoptosis (Fig. 9). The present study provided a scientific foundation and information for the use of CZ2HF for the treatment of AD.

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Availability of data and materials

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

Authors' contributions

QG and JS designed the experimental approaches. LZ performed all the other studies described herein, except the western blotting conducted by YD and JG. LZ and JG wrote the manuscript with the help from QG. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zunyi Medical University (Zunyi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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