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Apigenin attenuates acrylonitrile-induced neuro-inflammation in rats: Involved of inactivation of the TLR4/NF-κB signaling pathway

Fenxian Zhao[1], Yuhui Dang[1], Ruiping Zhang, Guangzhuang Jing, Weitao Liang, Li'ao Xie, Zhilan Li*

Institute of Maternal, Child and Adolescent Health, School of Public Health, Lanzhou University, Lanzhou 730000, PR China

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ABSTRACT

Acrylonitrile (ACN) is often found in the productions of synthetic fibers, rubber, and plastics. Exposure to ACN could cause pathological changes of the nervous system, which appeared early and were very serious. Current studies have found that the neurotoxicity is mainly related to oxidative damage and inflammation induced by ACN. Apigenin (AP) is a flavonoid subtype compound that is less toxic, non-mutagenic, and widely distributed in many types of vegetables and fruits. Studies have confirmed that it has nice antioxidant, anti-inflammatory and anti-apoptotic properties in the nervous system and related disease models, such as Alzheimer's disease. In this study, we used AP (117, 234 and 351 mg·kg^{-1}) pretreatment intragastrically to resist the neurotoxicity caused by ACN gavage (46 mg·kg^{-1}) for 28 days, and then detected the oxidative stress, inflammation mediated by the TLR4/NF-κB signaling pathway, and apoptosis to evaluate the protective effect of AP. The results showed that AP could lessen the autonomic activities of rats, and improve the abnormal morphology of neurons induced by ACN. AP could also reduce the oxidative stress, downregulate the TLR4/NF-κB signaling pathway, decrease the levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and inhibit the mitochondria-mediated neuron apoptosis. Immunofluorescence result showed that AP could decrease the activation and nuclear transfer of NF-κB induced by ACN. These results suggested that AP could protect the brain against ACN-induced neurotoxicity by inhibiting the TLR4/NF-κB signaling pathway and could exhibit a neuroprotective effect.

1. Introduction

Acrylonitrile (ACN), an important monomer in the organic synthesis industry, is widely used in the productions of synthetic fibers, resins, and plastics [1, 2]. ACN has been found in drinking water, food, cigarette smoke, and air [3]. ACN is absorbed orally, by vapor inhalation, and through intact skin [4]. The exposure to ACN for humans is often through acute incidental exposure at a high level, or by chronic lasting exposure at a low level [5]. Acute exposure to ACN could cause myasthenia of the limbs, labored or irregular breathing, dizziness, misjudgment, hallucinations, cyanochroia, nausea, collapse, loss of consciousness, convulsions, and death [2, 6]. Chronic exposure caused disorder of autonomic nervous functions such as lowered arterial pressure, unstable pulse, diffuse dermographia, increased sweating, and alteration in orthostatic reflex [2]. There are two pathways for ACN to metabolize within the body: glutathione (GSH) conjugation and cytochrome P450 2E1 (CYP 2E1) oxidation [7, 8]. Both pathways can increase the oxidative stress load and then induce the peroxidation of biofilm lipids, nucleic acids, and protein through GSH depletion, free radical increase, and CN^- release. The nervous system is extremely sensitive to oxidative stress, and brain is also a vital target of ACN toxicity [10]. A study reported that ACN could induce mitochondrial dysfunction in the rat hippocampus, mainly expressed as the increase of malondialdehyde (MDA) and the decreases of manganese-superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), and GSH [11]. When the rats were subchronically exposed to ACN, the expression of inducible nitric oxide synthase (iNOS) and the nitric oxide (NO) content were promoted in the brain, oxidative stress was triggered, and then the p38 MAPK signaling pathway was activated, in turn aggravating brain injury [12]. Previous studies have shown that ACN caused edema and degeneration of nerve cells, vacuolation of neurons in the hippocampus, and widening of the interspace around brain vessels in the rat; at the same time, disordered myelin sheaths, malformed neuronal nuclei, and chromatin condensation were all observed in the periphery of the nucleus [13]. In addition, a study has proved that inflammation, initiated by reactive oxygen

* Corresponding author.
E-mail address: lizhl@lzu.edu.cn (Z. Li).
[1] Equal contributors.

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species (ROS) and induced by nuclear transcription factor κB (NF-κB), was an important event in ACN-induced neurotoxicity in vitro [14]. Circulating pro-inflammatory cytokines [interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α)] were associated with the activation of extrinsic apoptotic pathways, and apoptosis has been observed in neurons exposed to ACN [15, 16]. These studies have suggested that oxidative stress might be the primary agent of ACN neurotoxicity. In view of this, there has been an increasing interest in the preventions and therapeutic possibilities of potential antioxidants against neurotoxicity induced by ACN.

Apigenin (AP), 4′, 5, 7-trihydroxyflavone, belongs to the flavonoid subgroup, and widely distributes in various kinds of plants and vegetables [17, 18]. It is especially rich in parsley (*Petroselinum crispum*) and celery (*Apium graveolens*) [19]. AP is a less toxic and non-mutagenic substance that has recently received much attention in the researches of the nervous system and related diseases [20]. Our study found that AP (234 and 468 $mg{\cdot}kg^{-1}$) showed significant antioxidation in the brain of Sprague-Dawley (SD) rats [21]. In another study, AP (350, 700, and 1400 $mg{\cdot}kg^{-1}$) reduced the levels of blood lead and brain lead, and lessened lipid peroxidation in the mouse brain exposed to lead acetate by improving SOD and catalase (CAT), and decreasing MDA and lipid peroxide (LPO) [22]. There were some other studies in vitro and in vivo demonstrating that AP had obvious anti-inflammatory, antioxidant, and anti-apoptotic effects [23] Smolinski et al. reported that AP (50 $mg{\cdot}kg^{-1}$) could reduce the increase of interleukin-6 (IL-6) and TNF-α induced by lipopolysaccharide (LPS) in mouse serum [24]. In 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced Parkinsonian mouse model, it was found that AP had a neuroprotective effect mainly due to its antioxidant activity (increased SOD, CAT, and GSH and decreased LPO), and prevented the decreases of tyrosine hydroxylase (TH) and brain-derived neurotrophic factor (BDNF) as well as the increases of glial fibrillary acidic protein (GFAP) and TNF-α [25]. In a human induced pluripotent stem cell (iPSC)-derived model of Alzheimer's disease (AD), AP obviously downregulated the activity of Caspase-3/7, and thus prevented apoptosis [26]. The bioactivity of AP is related to the chemical structure (Fig. 1): the hydroxyl can combine with free radicals and chelate metal ions, and the C_2-C_3 can stabilize structure [27].

Bueno et al. have demonstrated that Toll-like receptor 4 (TLR4), an innate immune receptor, might play a significant role in inflammation occurred in neuropsychiatric diseases [28]. It has been found that TLR4 was crucial to the CNS toxicity induced by neurotoxin, such as MPTP, carbon monoxide (CO), and diazinon [29–31]. The relationship between ACN and TLR4 have not been observed in previous studies, therefore, we speculated that ACN-induced neurotoxicity may be related to TLR4. The purpose of the present study is to clarify whether AP has a protective effect against the neurotoxicity induced by ACN via inhibiting neuro-inflammation mediated by the TLR4/NF-κB signaling pathway, and thus to search for an effective substance to prevent and treat neurotoxicity of ACN.

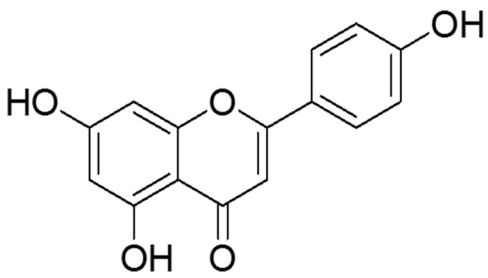

Fig. 1. Chemical structure of AP.

Table 1
Primer sequences of genes.

Genes	Primers	Sequences (5′-3′)
TLR4	Forward	5′-CTCACAACTTCAGTGGCTGGATTTA-3′
	Reverse	5′-GTCTCCACAGCCACCAGATTCTC-3′
IKK-α	Forward	5′-GGTCATCTAATGTCCCAGCCTTC-3′
	Reverse	5′-CTCCATCTGTAACCAGCTCCAGTC-3′
IκB-α	Forward	5′-TGACCATGGAAGTGATTGGTCAG-3′
	Reverse	5′-GATCACAGCCAAGTGGAGTGGA-3′
NF-κB	Forward	5′-CGACGTATTGCTGTGCCTTC-3′
	Reverse	5′-TTGAGATCTGCCCAGGTGGTA-3′
Bax	Forward	5′-CATTGACACCAAAGAGTACGC-3′
	Reverse	5′-TGTTGATGAATCTCAGCAGGA-3′
Bcl-2	Forward	5′-GACTGAGTACCTGAACCGGCATC-3′
	Reverse	5′-CTGAGCAGCGTCTTCAGAGACA-3′
Caspase-9	Forward	5′-CTGAGCCAGATGCTGTCCCATA-3′
	Reverse	5′-GACACCATCCAAGGTCTCGATGTA-3′
Caspase-3	Forward	5′-GAGACAGACAGTGGAACTGACGATG-3′
	Reverse	5′-GGCGCAAAGTGACTGGATGA-3′
β-actin	Forward	5′-GGAGATTACTGCCCTGGCTCCTA-3′
	Reverse	5′-GACTCATCGTACTCCTGCTTGCTG-3′

2. Materials and methods

2.1. Animal preparation and administration

Sixty of healthy adult male SD rats of SPF-level (180–220 g) were purchased from the Medical Laboratory Animal Centre of Gansu University of Chinese Medicine, bred in the experiment center of the School of Public Health of Lanzhou University, and fed on standard pellet chow and water ad libitum. All the experiments were approved and conducted according to the guidelines of the ethics committee of the School of Public Health of Lanzhou University.

The rats were randomly divided into five groups (12 per group): (1) control group (corn oil), (2) ACN-treated group (46 $mg{\cdot}kg^{-1}$ ACN), (3) AP1 group (46 $mg{\cdot}kg^{-1}$ ACN + 117 $mg{\cdot}kg^{-1}$ AP), (4) AP2 group (46 $mg{\cdot}kg^{-1}$ ACN + 234 $mg{\cdot}kg^{-1}$ AP), and (5) AP3 group ($mg{\cdot}kg^{-1}$ ACN + 351 $mg{\cdot}kg^{-1}$ AP). The dosage was determined according to previous studies conducted by our research team [32].

ACN (Tianjin Kaixin Chemical Co., Ltd. China, 99% pure) and AP (Shaanxi Ci Yuan Biotechnology Co., Ltd. China, 98% pure) were dissolved and diluted in corn oil. AP was administered intragastrically 0.5 h before ACN gavage, daily for 28 days. An equal of volume of corn oil served as the control group.

2.2. Measurement of brain wet weight and organ coefficient

After the rats were euthanized, the brain were removed and weighed. The percentage of wet weight was calculated according to the following formula: organ coefficient (%) = (wet weight/body weight) × 100%.

2.3. Detection of autonomic activity of rats (open field test, OFT)

At the beginning and ending of treatment, the autonomic activity of rats was detected using the VideoTrack ver. 3.0 rodent behavior tracking software (Viewpoint Life Sciences Co., Ltd. France). The test was performed in a quiet and dark room. Four rats were put into four separate regional centers every time and operated according to the instruction manual of the system. The test lasted for 20 min, and each rat was permitted to explore freely for 10 min. The total distance of motion in the latter 10 min was analyzed to reflect autonomic activity.

2.4. Pathological brain examination

Three polyoxymethylene-fixed, paraffin wax-embedded brain samples from each group were sectioned at 5 μm and stained with

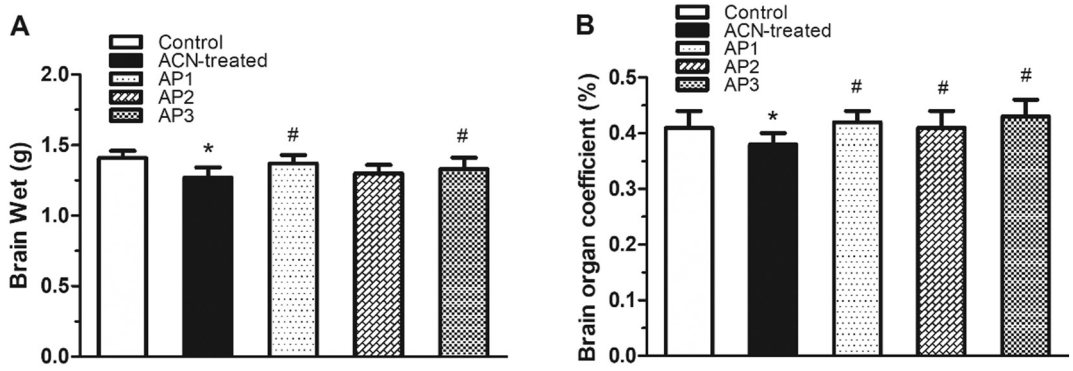

Fig. 2. Effect of AP on (A) brain wet and (B) brain organ coefficient in rats after ACN treatment ($n = 12$). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

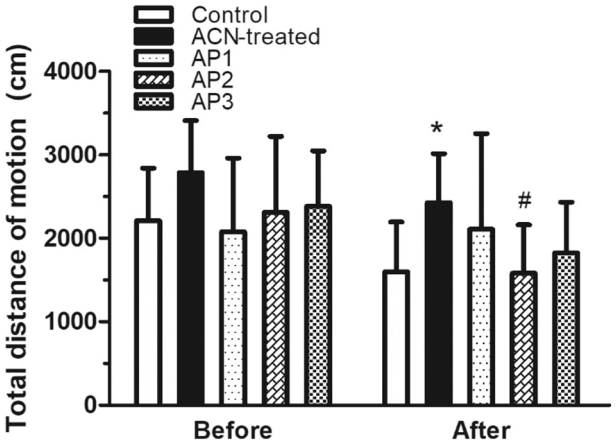

Fig. 3. Effect of AP on autonomic activities of rats before and after ACN treatment ($n = 12$). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

hematoxylin-eosin. The histological diagnoses were determined by two independent pathologists. The neurons in the cerebral cortex and hippocampus were examined by light microscope.

2.5. Determination of oxidative stress markers

The activities of SOD and GSH-Px as well as the contents of GSH and MDA in the rat brain were measured using commercial kits (Nanjing Jiancheng co., China). Protein concentrations were determined using the BCA Protein Assay Kit.

2.6. Western blot analysis

Brain samples were lysated on ice with radioimmunoprecipitation (RIPA) lysis buffer and then centrifuged (12,000*g*, 15 min, 4 °C). Protein concentrations were determined using the BCA Protein Assay Kit. Equal amounts of protein (60 μg) per lane were separated by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and transferred to a polyvinylidene-difluoride (PVDF) membrane. The membrane was blocked in 5% nonfat dry milk for 2 h at room temperature and incubated overnight at 4 °C with primary antibodies against HMGB-1 (1:2000; Elabscience Biotechnology, China), TLR4 (1:1000; Cell Signaling Technology, USA), IKK-α (1:1000; Cell Signaling Technology, USA), p-IKK-α/β (1:1000; Cell Signaling Technology, USA), IκB-α (1:1000; Cell Signaling Technology, USA), p-IκB-α (1:1000; Cell Signaling Technology, USA), NF-κB p65 (1:1000; Cell Signaling Technology, USA), Bax (1:1000; Signalway Antibody, USA), Bcl-2 (1:1000; Signalway Antibody, USA), Cyt-c (1:1000; Cell Signaling Technology, USA), Caspase-9 (1:500; Signalway Antibody, USA), Caspase-3 (1:1000; Cell Signaling Technology, USA), and GAPDH (1:5000; Signalway Antibody, USA) in Tris-buffered saline with Tween 20 (TBST). Then, the membrane was washed with TBST five times for 5 min each time. The membrane was processed with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The protein bands were visualized with ECL, and images were acquired. The protein band densities were quantified using Image J software (National Institutes of Health, USA). GAPDH served as an endogenous control. The brain tissues of six animals in each group were used for Western blot analysis. All experiments were repeated at least three times.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the rat brain with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and analyzed by qRT-PCR. *β-actin* served as an endogenous control. Rat-specific primers for *TLR4*, *IKK-α*, *IκB-α*, *NF-κB*, *Bax*, *Bcl-2*, *Caspase-9*, and *Caspase-3* were synthesized by Takara Bio (Japan), and the sequences were shown in Table 1. The melting curve, which was measured after amplification, showed a single product peak, indicating good product specificity.

2.8. Immunofluorescence

Three of the rat brains in each group were examined for the activation and nuclear transfer of NF-κB p65 with the NF-κB Activation-Nuclear Translocation Assay Kit (Beyotime Biotechnology co., China). Five visual fields of each rat brain were observed to count NF-κB-positive cells by fluorescence microscopy, and the image superposition was done using Image J software.

2.9. Test of neuron apoptosis

Three of the rat brains in each group were examined for neuronal apoptosis with the transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method (Wuhan Boster biology Co., Ltd. China). Five visual fields of each rat brain were examined to determine the amount and distribution of apoptotic neurons using a light microscope.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Measurements of contents of IL-6, IL-1β, and TNF-α in rat brain were done by ELISA assay with a commercial kit (Elabscience Biotechnology co., China).

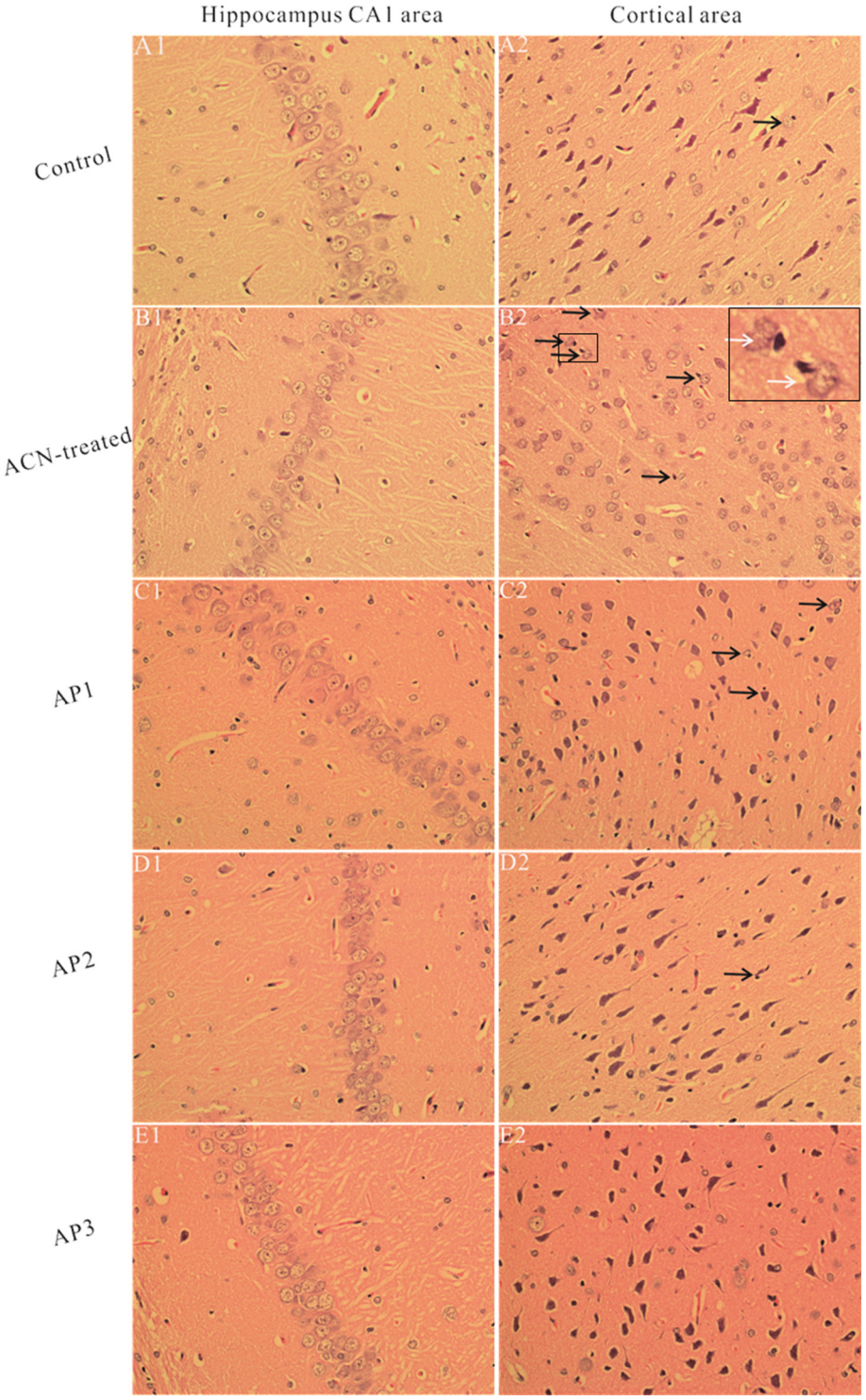

Fig. 4. Effect of AP on pathological morphology of neurons in the hippocampus and cortex in the rat brain after ACN treatment ($n = 3$). Arrows indicated neuronophagia. Magnification ×400.

2.11. Statistical analysis

All data were presented as mean ± standard deviation (SD). SPSS 20.0 (IBM, USA) was used for statistical analysis. All data were subjected to one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of AP on brain wet weight and organ coefficient

During the experimental period, there was no statistical significance in the changes of body weight ($P > 0.05$), although the weights of all rats showed an upward trend. As shown in Fig. 2A, the brain wet weight in the ACN-treated group was significantly lower than that in the control group ($P < 0.05$, Fig. 2A), and then AP1 and AP3 improved the

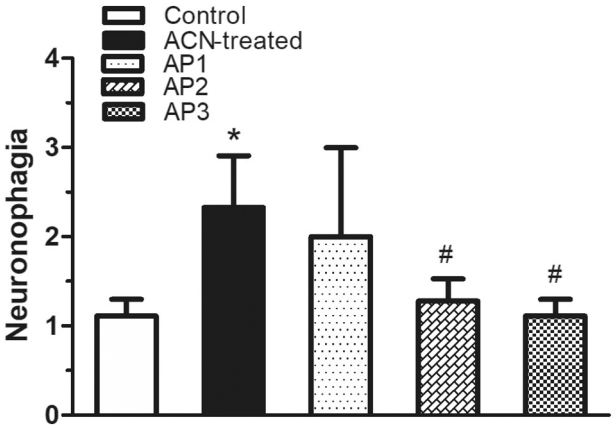

Fig. 5. Effect of AP on neuronophagia of the cortex in the rat brain after ACN treatment (n = 3). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

brain wet weight compared with the ACN-treated group ($P < 0.05$, Fig. 2A). Compared with the control group, ACN induced a significant decrease of brain organ coefficient ($P < 0.05$, Fig. 2B), but the decrease was significantly attenuated by AP ($P < 0.05$, Fig. 2B).

3.2. Effect of AP on autonomic activities of rats

The autonomic activities of rats were checked by OFT. Before the exposure, there was no significant difference in the total distance of motion among groups ($P > 0.05$, Fig. 3). However, the autonomic activity in the ACN-treated group was increased compared with the control group after the exposure ($P < 0.05$, Fig. 3), but this increase was significantly reduced by AP2 ($P < 0.05$, Fig. 3).

3.3. Effect of AP on pathological morphology

As shown in Fig. 4, the neurons in the control group were almost normal with the complete structure of pyramidal cells in the cortex and neatly arranged neurons in the hippocamcus CA1 area. However, ACN induced obvious structural changes of neurons in the hippocampus and cortex, including the pale nuclei and cytoplasm, reduction of nerve cell layer in the hippocampus, and the appearance of neuronophagia (indicated by arrows) in the cortex. AP pretreatment alleviated these changes induced by ACN manifested as the structure close to normal of pyramidal cells in the cortex, and the increase and neatly arrangement of nerve cell layers in hippocamcus, most obviously in the AP2 group.

By comparing the number of neuronophagia among groups, it was found that AP2 and AP3 could significantly reduce the increased number triggered by ACN ($P < 0.05$, Fig. 5).

3.4. Effect of AP on oxidative stress

The content of MDA reflects the peroxidation damage degree of biofilm lipids, whereas the levels of GSH, SOD, and GSH-Px indicate the ability of organisms against oxidation. As shown in Fig. 6A, the content of MDA in the ACN-treated group was significantly higher than that in the control group ($P < 0.05$, Fig. 6A). The light decrease of GSH caused by ACN was significantly improved by AP ($P < 0.05$, Fig. 6B). AP also increased the level of GSH-Px compared with the ACN-treated group ($P < 0.05$, Fig. 6C). And, the slight reduction of SOD was significantly improved by AP2 ($P < 0.05$, Fig. 6D).

3.5. Effect of AP on the TLR4/NF-κB signaling pathway

To demonstrate whether AP was involved in the TLR4/NF-κB signaling pathway, the expressions of HMGB-1, TLR4, IKK-α, p-IKK-α/β, IκB-α, p-IκB-α, and NF-κB p65 were examined. As shown in Fig. 7A and

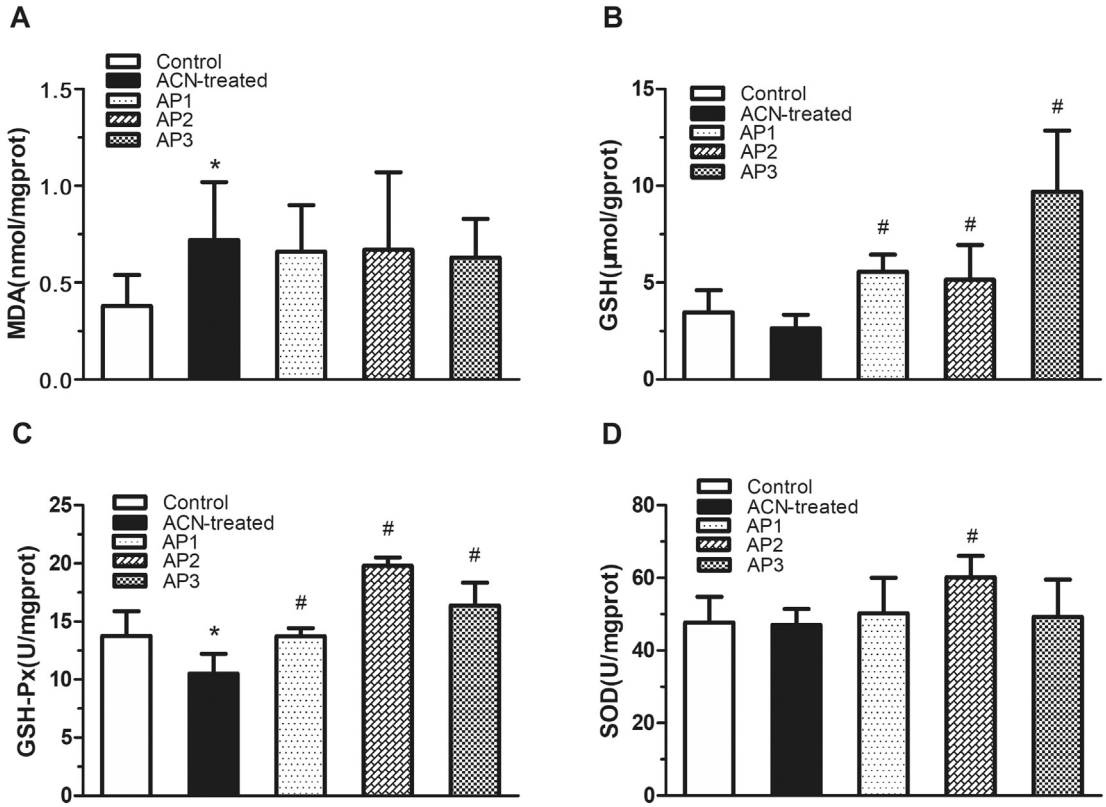

Fig. 6. Effect of AP on (A) MDA content, (B) GSH content, (C) GSH-Px activity, and (D) SOD activity in the rat brain after ACN treatment ($n = 6$). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

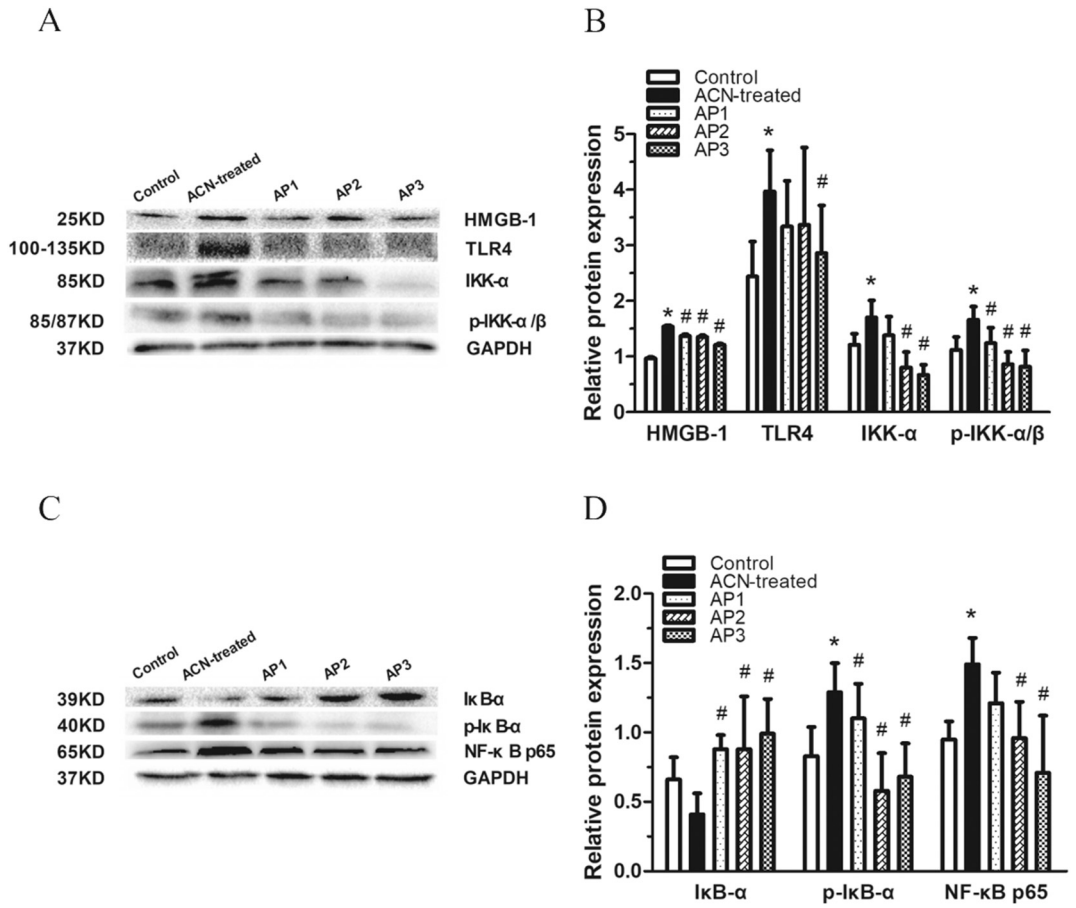

Fig. 7. Effect of AP on the protein expression of the TLR4/NF-κB signaling pathway in the rat brain after ACN treatment ($n = 6$). (A) Representative immunoblots of the expression of HMGB-1, TLR4, IKK-α, and p-IKK-α/β. (B) Western blot analyses of HMGB-1, TLR4, IKK-α, and p-IKK-α/β. (C) Representative immunoblots of the expression of IκB-α, p-IκB-α, and NF-κB p65. (D) Western blot analyses of IκB-α, p-IκB-α, and NF-κB p65. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

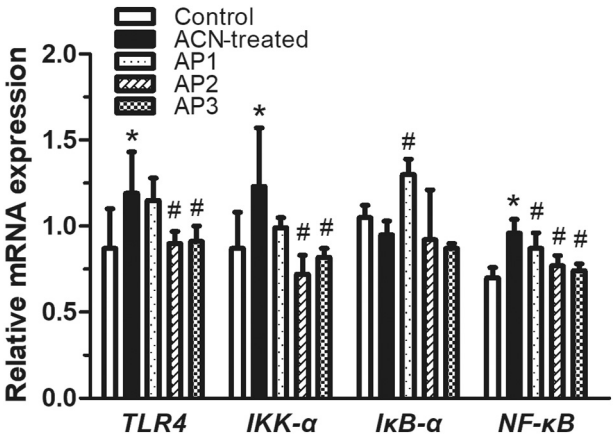

Fig. 8. Effect of AP on the mRNA expression of the TLR4/NF-κB signaling pathway in the rat brain after ACN treatment (n = 6). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

C, the findings indicated that ACN could induce dramatic increases of HMGB-1, TLR4, IKK-α, p-IKK-α/β, p-IκB-α, and NF-κB p65. IκB-α, which is the activation indicator of NF-κB, is often detected to determine the activation of NF-κB. Results showed that ACN could decrease the expression of IκB-α. However, in all AP groups, all of them were largely restored. And, the mRNA expressions of these factors were consistent with protein expressions. Data are shown in Fig. 7B, D and Fig. 8. In addition, the ratios of p-IKK-α/β/IKK-α and p-IκB-α/IκB-α also indicated that NF-κB was activated by ACN, but this activation was inhibited by AP pretreatment (Fig. 9A and B).

3.6. Effect of AP on NF-κB activation and nuclear transfer

The anti-NF-κB p65 antibody was fluorescently red by the Cy3-labeled secondary antibody, which was used to detect cellular subunits of NF-κB (p65). The nuclei were stained with blue fluorescence [stained by diamidino-phenyl-indole (DAPI)] (Fig. 10). In the control group, very few neurons were labeled with Cy3, but there were many in the ACN-treated group, and the NF-κB p65 subunit was significantly activated and transferred to the nucleus, indicated by the arrows in the merged figure (Fig. 10, right). However, in all AP groups, the neurons stained red were decreased, especially in the nuclei, indicating that AP might partially inhibit the nuclear transfer of NF-κB.

The cell count result as shown in Fig. 11, ACN could cause a significant increase of NF-κB-positive cells ($P < 0.05$), but AP pretreatment attenuated this increase markedly ($P < 0.05$).

3.7. Effect of AP on pro-inflammatory cytokines

The content of IL-6 in the ACN-treated group was significantly higher than that in the control group ($P < 0.05$, Fig. 12), whereas in all AP groups, IL-6 was significantly decreased ($P < 0.05$, Fig. 12). Compared with the control group, IL-1β and TNF-α in the ACN-treated group were not significantly changed. But, compared with the ACN-treated group, the content of TNF-α in the AP1 group was decreased ($P < 0.05$, Fig. 12). Moreover, the content of TNF-α was promoted in

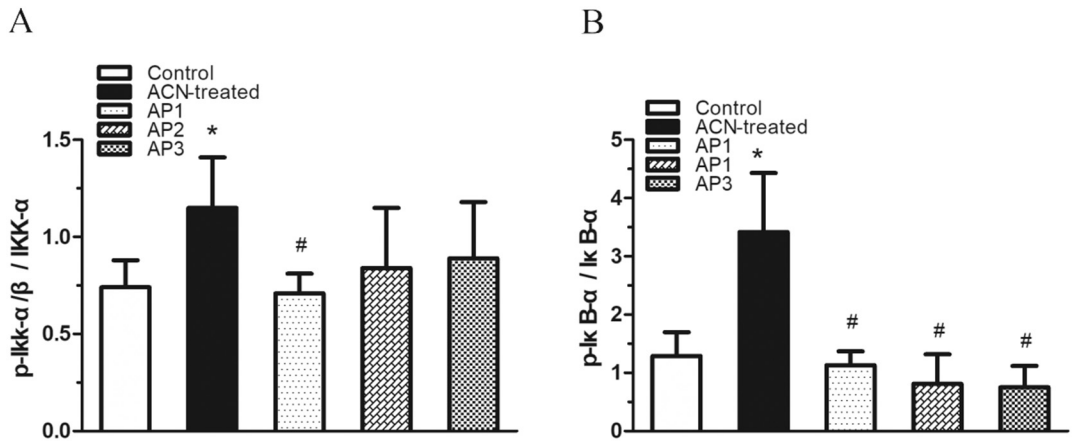

Fig. 9. Effect of AP on (A) p-IKK-α/β/IKK-α and (B) p-IκB-α/IκB-α in the protein expression in the rat brain after ACN treatment (n = 6). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

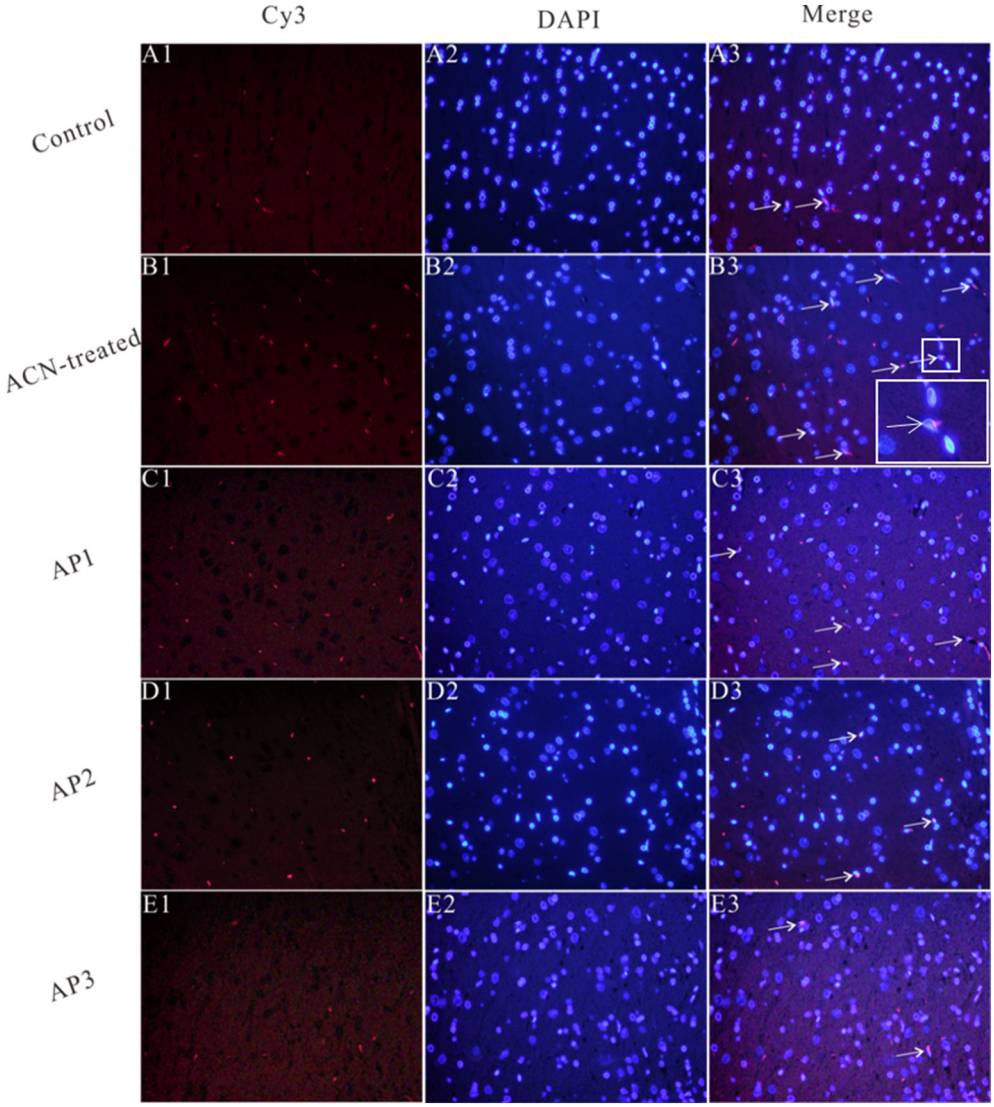

Fig. 10. Effect of AP on the activation and nuclear transfer of NF-κB in neurons after ACN treatment ($n = 3$). NF-κB p65 was labeled with Cy3 (red) and the nucleus was stained with DAPI (blue). Arrows indicated that NF-κB p65 was activated and transferred into the nucleus. Magnification ×400. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

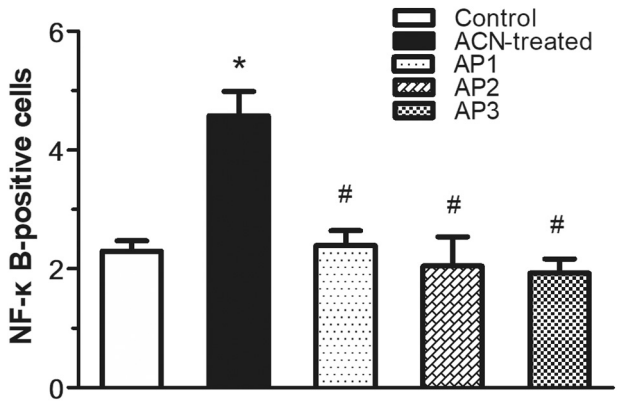

Fig. 11. Effect of AP on NF-κB-positive neurons after ACN treatment (n = 3). Magnification ×400. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

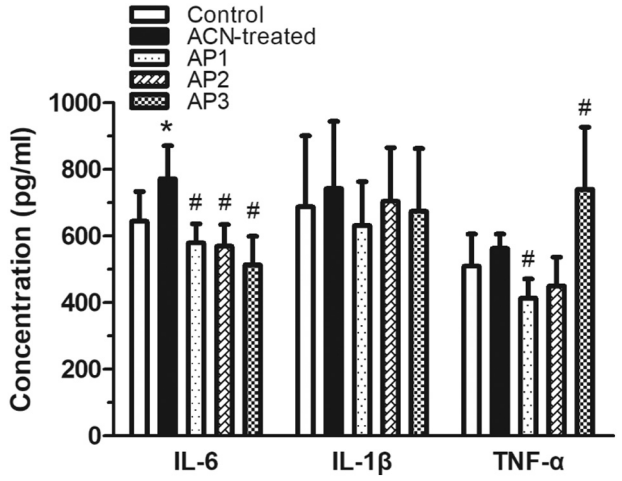

Fig. 12. Effect of AP on the contents of IL-6, IL-1β, and TNF-α in the rat brain after ACN treatment ($n = 6$). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

the AP3 group ($P < 0.05$, Fig. 12), which required further exploration.

3.8. Effect of AP on neuronal apoptosis

The neuronal apoptosis in each group was shown in Fig. 13 (A, B, C, D, and E). By comparing the number of TUNEL-positive cells, it was found that the number of apoptotic cells in the ACN-treated group were significantly more than that in the control group ($P < 0.05$, Fig. 13), which could be significantly reduced by AP3 pretreatment ($P < 0.05$, Fig. 13).

3.9. Effects of AP on cell apoptosis-relevant proteins

Finally, we assessed the expressions of several apoptosis-related factors including Cyt-c, Bax, Bcl-2, Caspase-9, and Caspase-3. As shown in Fig. 14A, we found that ACN induced the increases of Cyt-c, Bax, Caspase-9, and Caspase-3. Their mRNA expressions were consistent with protein expressions. The data are shown in Fig. 14B, C, D, and E. To some extent, these changes could be restored by AP.

In family proteins of Bcl-2, Bcl-2 carries out anti-apoptotic effects, whereas Bax promotes apoptosis. To a certain extent, the ratio of Bcl-2/Bax determines whether or not cells undergo apoptosis. As shown in Fig. 14F, the ratio of Bcl-2/Bax in protein expression in the ACN-treated group were significantly lower than that in the control group ($P < 0.05$, Fig. 14D), but this decrease was improved by AP pretreatment, especially in the AP3 group.

4. Discussion

Researches have shown that ACN could cause dysfunction of multiple systems and organs, such as immunotoxicity [33] and neurotoxicity [34], and that ACN possesses special adverse effects including carcinogenesis [35], embryotoxicity [36], and mutagenesis [37]. Several studies have proved that AP played a protective role against the damage caused by many harmful factors, such as ameliorating chronic mild stress-induced depressive behavior in rats [38], attenuating copper-mediated β-amyloid neurotoxicity in an AD cell model [39], and suppressing ACN-induced inflammation and apoptosis in testicular cells [40]. Our experimental results found that ACN decreased brain wet weight and organ coefficient, increased autonomic activities of rats consistent with previous studies [41, 42], and altered the normal morphology of neurons in the hippocampus and cortex. However, AP pretreatment significantly restored these abnormal changes, and presented a neuroprotective effect.

ACN can be oxidized to form 2-cyano ethylene oxide (CEO) by CYP 2E1, then CEO releases CN^- by hydrolysis. ACN and CEO are detoxified through GSH conjugation, which always causes GSH reduction or even exhaustion, then alters the cellular redox status and increases oxidative stress [43]. The oxidation of ACN by CYP 2E1 induces the production of more free radicals due to the permeability characteristic of the enzyme. Finally, CN^-, a typical metabolic inhibitor neurotoxin, possesses strong oxidative toxicity, which selectively damages dopaminergic neurons and causes Parkinson's disease-like neuropathy [44]. In addition, brains are extremely sensitive to oxidative damage; therefore, ACN-induced neurotoxicity often occurs early and is serious. AP, a potent antioxidant, has attracted considerable attention in the study of neurological and related disease models in recent years [23]. A study reported that AP could suppress oxidative stress by directing free radical scavenging action and upregulating intracellular antioxidant defense in an AD model [45]. Another research found that AP resisted the excitotoxicity induced by kainic acid though reducing ROS and inhibiting GSH consumption [46]. In the present study, we detected the levels of MDA, GSH, SOD, and GSH-Px in the rat brain to indicate oxidative damage degree and antioxidant capacity. The results found that ACN increased the content of MDA and decreased the activity of GSH-Px, whereas AP markedly improved the levels of GSH and GSH-Px, which was consistent with a study by El-Sayed et al. that hesperidin may have a beneficial role against ACN-induced oxidative stress in the brain [47]. The not obviously reduction of SOD by ACN, which may be related to the treatment time, was consistent with the result of research on ACN-induced reproductive damage conducted by Dang et al. [48]. It suggested that AP could reduce oxidative stress caused by ACN, and play a corresponding antioxidant role, mainly affecting a series of reactions involving GSH.

Both ACN exposure and metabolism can generate ROS, which can initiate inflammatory responses [14]. This is mainly achieved by activating NF-κB transcription factors. Multiple harmful factors such as ROS and free radicals can activate NF-κB, primarily though activating the upstream kinases (IKK, PI3K, AKT, MEKK-1). And, it is involved of the phosphorylation of the NF-κB complex in the cytoplasm, and then used in the nucleocytoplasm to promote induction of downstream related gene transcription [49]. TLR4 belongs to the super-family of pattern recognition receptors (PRRs), which can detect LPS via pathogen-associated molecular patterns (PAMPs) and identify cellular or tissue danger/damage signals (fibrinogen, HSP60–70, and HGMB-1) via damage-associated molecular patterns (DAMPs), and then initiate innate inflammation. After binding to the corresponding ligand, TLR4 activates IKK through a variety of ways, and then activates NF-κB [28]. IκB-α is an important inhibited factor in activating NF-κB. Usually, NF-κB combines with IκB-α in the cytoplasm with no transcription activity. When the upstream pathway (IKK) is activated, IκB-α is subsequently

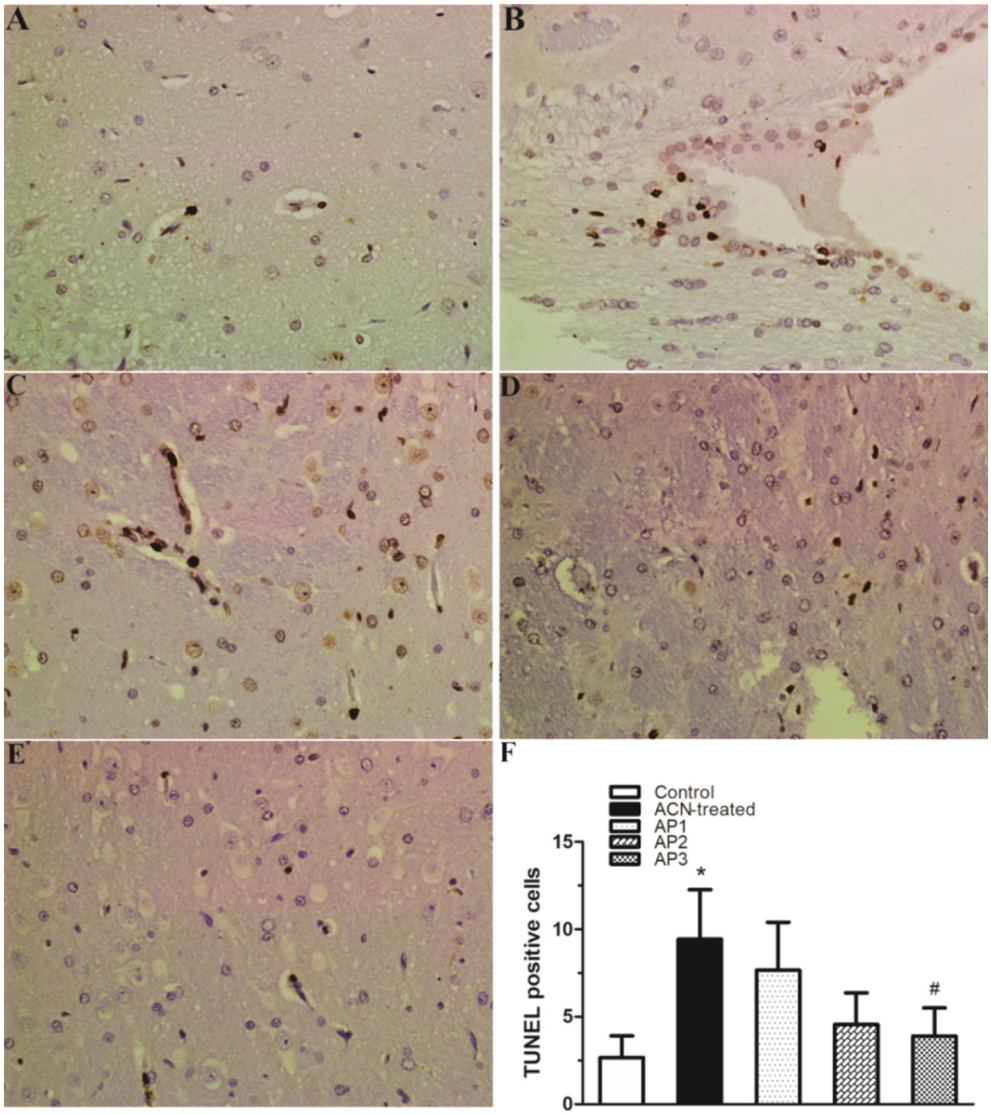

Fig. 13. Effect of AP on neuronal apoptosis in the rat brain after ACN treatment ($n = 3$). (A) Control group, (B) ACN-treated group, (C) AP1 group, (D) AP2 group, (E) AP3 group, and (F) Comparison of TUNEL-positive cells. Magnification ×400. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

degraded by phosphorylation, which promotes the nuclear transfer of NF-κB and induces upregulation of gene expressions such as pro-inflammatory cytokines, adhesion molecules, and chemokines [50]. Zhang et al. found that AP played a neuroprotective role in subarachnoid haemorrhage rats mainly manifested by inhibiting the TLR4/NF-κB signaling pathway, reducing the production of pro-inflammatory factors [iNOS, cyclooxygenase-2 (COX-2), IL-6, IL-1, and TNF-α], and suppressing inflammation [51]. In our results, AP significantly downregulated the activation of the TLR4/NF-κB signaling pathway, decreased the levels of pro-inflammatory factors on a certain extent, and inhibited the neuro-inflammation caused by ACN. In addition, the immunofluorescence result showed that AP could reduce the activation and nuclear transfer of NF-κB p65 induced by ACN.

The activation and nuclear transfer of NF-κB can cause inflammation, which involves of changes in microcirculation and of aggregation of inflammatory cells (macrophages and granulocytes). When macrophages secrete inflammatory cytokines including acute (IL-6, IL-1, and TNF-α) and chronic cytokines [transforming growth factor-β (TGF-β)], the inflammatory responses are initiated. These cytokines stimulate inflammatory mediators released from stromal cells, such as endothelial cells and fibroblasts, and cause local microvascular dilatation and increased capillary permeability. This, in turn, promotes leukocyte aggregation in inflammatory areas by releasing chemokines and expressing cell adhesion molecules [52]. A study reported that AP could reduce the levels of IL-1β, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) in rat serum with spinal cord injury [53]. Our results found that ACN could induce significant increase of IL-6, and slight increase of IL-1β and TNF-α in the rat brain while AP1 reduced the levels of IL-6 and TNF-α. The increase of TNF-α in AP3 group might be due to the inhibited protective effect of AP because of the excessive dosage. Moreover, the pro-inflammatory factors (IL-1 and TNF-α) also activate IKK by other pathways, thereby resulting in sustained or amplified inflammatory response via causing the activation of NF-κB and triggering a cytokine cascade reaction [14].

In the process of inflammation, the accumulation of macrophages and leukocytes in the injured sites also produces a large number of free radicals, which further aggravates brain damage in addition to the oxidative damage induced by ACN metabolism. One of the results is the peroxidation of biofilm lipids including the mitochondrial membrane, which increases membrane permeability and degrades membrane lipids [52]. The changes of mitochondrial membrane permeability always lead to the collapse of the mitochondrial membrane potential (involving of the mitochondrial permeability transition, MPT), and then elicit the release of Cyt-c and the activation of Caspase-9 and Caspase-3 [54,

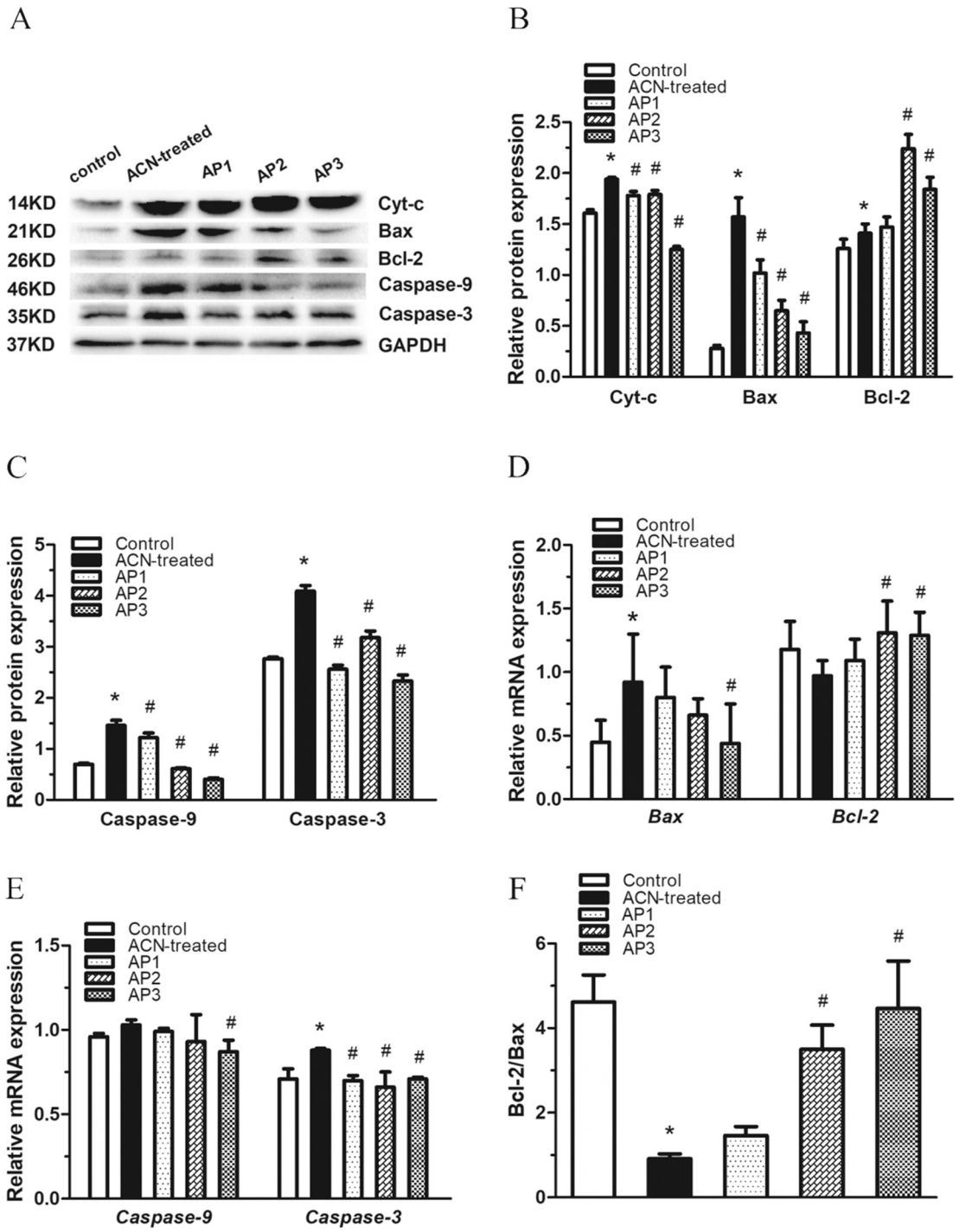

Fig. 14. Effect of AP on the expression of apoptosis-relevant proteins in the rat brain after ACN treatment ($n = 6$). (A) Representative immunoblots of these proteins' expression. (B) Western blot analyses of Cyt-c, Bax, and Bcl-2. (C) Western blot analyses of Caspase-9 and Caspase-3. (D) The mRNA expressions of *Bax* and *Bcl-2*. (E) The mRNA expressions of *Caspase-9* and *Caspase-3*. (F) The ratios of Bcl-2/Bax in protein expression. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. ACN-treated group.

55], which is called the mitochondria-mediated apoptosis pathway and is the main pathway of programmed cell death in mammals. When cells are stimulated by apoptosis signals, the executor of apoptosis, Caspase-3, is activated via the release of Cyt-c from mitochondria into the cytoplasm and then the combination with Apaf-1 and Caspase-9 to form apoptotic bodies, finally activating caspase-9. Gene knockout experiments have confirmed that apoptosis can be transmitted through the Cyt-c-Apaf-1-Caspase-9-Caspase-3 dependent pathway [56, 57]. Besides, mitochondrial release of apoptosis factors are regulated by the Bcl-2 family, which consist of anti-apoptotic factors (Bcl-2, Bcl-XL, Bcl-w) and pro-apoptotic factors (Bax, Bid, Bak). The ratio of Bcl-2/Bax determines whether or not the cell undergoes apoptosis to a certain extent [58]. Our results found that ACN upregulated expressions of Cyt-c, Bax, Caspase-9, and Caspase-3, and decreased the ratio of Bcl-2/Bax. However, pretreatment with AP significantly mitigated these changes and exhibited an anti-apoptotic effect expressed as downregulating expressions of Cyt-c, Bax, Caspase-9, and Caspase-3, increasing the expression of Bcl-2 and the ratio of Bcl-2/Bax. The result of TUNEL assay also showed that ACN significantly induced neuronal apoptosis, which was alleviated by AP supplementation.

In summary, AP could protect rats' brains against the neurotoxicity induced by ACN mainly expressed as reducing autonomic activities of rats, and restoring the normal morphology of neurons in the hippocampus and cortex. With respect to underlying mechanisms of the effects of AP, it might play a neuroprotective role by inhibiting oxidative stress, downregulating the TLR4/NF-κB signaling pathway, decreasing the inflammatory factors, suppressing neuronal apoptosis, and then showing antioxidant, anti-inflammatory, and anti-apoptotic characteristics. However, we failed to detect specific markers of central nervous system (CNS) injury [such as neuron-specific enolase (NSE) and GFAP], and failed to use specific blockers of NF-κB in vitro to evaluate the effect of AP, which should be involved in future researches.

Declaration of Competing Interest

None.

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