Article

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N-Acetylcysteine Alleviates Cerebral Ischemia-Reperfusion Injury by Inhibiting the JNK/Caspase-3 Signaling Pathway

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Background: Cerebral ischemia-reperfusion injury (CIRI) is a common neurological disorder involving pathways such as cell apoptosis and inflammation. N-acetylcysteine (NAC), an antioxidant and anti-inflammatory agent, is widely studied for various diseases. However, its specific mechanism in CIRI remains unclear. Therefore, this study aims to explore the mechanism of action of NAC in CIRI.

Methods: In this study, we utilized a Middle Cerebral Artery Occlusion (MCAO) mouse model to investigate the effects of N-acetylcysteine (NAC) on cerebral ischemia-reperfusion injury (CIRI). The experimental mice were divided into two groups: the Model group and the NAC treatment group. The NAC was administered after the induction of MCAO. The therapeutic outcomes were assessed through behavioral tests and neuropathological examinations. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was used to measure the levels of inflammatory factors, specifically tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Western blot analysis was employed to assess the expression of apolipoprotein E (APOE) following CIRI. The infarct volume post-CIRI was determined using pathological sections and image analysis. Additionally, the impact of NAC on the c-Jun N-terminal Kinase/Cysteine-aspartic Acid Protease-3 (JNK/Caspase-3) signaling pathway in neuronal cells was examined through immunofluorescence and western blot analysis.

Results: NAC treatment improved motor and cognitive functions ($p < 0.05$), reduced neuronal damage, and increased survival rates ($p < 0.05$). NAC treatment also led to a decrease in inflammatory factors, including TNF-α, IL-1β, and IL-6 after MCAO ($p < 0.01$). Furthermore, NAC significantly decreased the expression of APOE ($p < 0.01$), which contributed to neuroprotection. The administration of NAC also reduced the infarct volume induced by MCAO ($p < 0.01$) and inhibited cell apoptosis ($p < 0.05$). Hematoxylin and eosin (HE) staining demonstrated that NAC treatment resulted in reduced structural damage in the hippocampal CA1 region. Immunofluorescence and western blot analyses showed that NAC suppressed the expression of JNK, p-c-Jun, Caspase-3, and Caspase-9 in the oxygen-glucose deprivation (OGD) neuronal cell model ($p < 0.01$).

Conclusions: The findings of this study suggest that NAC alleviates CIRI by inhibiting the JNK and Caspase-3 signaling pathways. This reveals the potential mechanism of NAC in the treatment of CIRI and provides a theoretical basis for its clinical application.

Keywords: NAC; ischemia-reperfusion injury; JNK signaling pathway; Caspase-3; neuroprotection

Introduction

Cerebral ischemia-reperfusion injury (CIRI) is a prevalent neurological disorder characterized by a transient interruption in cerebral blood flow followed by reperfusion, leading to neuronal damage and death [1,2]. The development of this disease is associated with multiple factors, including inflammation, oxidative stress, and cell apoptosis [3]. Notably, the c-Jun N-terminal Kinase/Cysteine-aspartic Acid Protease-3 (JNK/Caspase-3) signaling pathways are crucial in the onset and progression of the disease [4–6].

In the context of CIRI, there is an interaction between the JNK/Caspase-3 signaling pathway and inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and IL-6 [7,8]. The activation of the JNK signaling pathway, triggered by hypoxia and reperfusion injury, contributes to the onset of inflammation and cellular apoptosis [9,10]. Simultaneously, activated Caspase-3 triggers apoptosis in neuronal cells, thereby exacerbating brain damage [11,12]. Additionally, hypoxia and reperfusion injury induce the release of TNF-α, IL-1β, and IL-6, which in turn stimulate an inflammatory response, thereby aggravating the severity of brain damage [13]. Given the interaction and regulatory mechanisms

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among these factors, it is evident that they play crucial roles in the manifestation of CIRI [14]. Therefore, the inhibition of JNK and Caspase-3 signaling pathways emerges as a significant strategy for the prevention and treatment of CIRI [15,16].

N-acetylcysteine (NAC) is a widely used drug with multiple biological activities, including antioxidant, anti-inflammatory, and neuroprotective effects [17]. In recent years, increasing evidence has shown that NAC significantly alleviates ischemia-reperfusion injury and other diseases by inhibiting inflammation, highlighting its potential clinical applications [18,19]. The protective effects of NAC are primarily achieved through the following mechanisms: elimination of free radicals, as NAC acts as an antioxidant, scavenging free radicals in the body and preventing oxidative damage to cell membrane lipids, thereby protecting cells from injury [20,21]. Additionally, NAC can inhibit the generation and release of inflammatory mediators such as TNFα, IL-1β, and IL-6, thereby reducing inflammation [22]. NAC can also enhance the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), thereby enhancing the body's antioxidant capacity [22,23]. However, there are still several areas that require further investigation before NAC can be safely and effectively utilized in a clinical setting. These include determining the optimal dosage and administration time, assessing long-term safety and tolerability, and exploring potential interactions with other medications.

In summary, this study hypothesizes that NAC alleviates CIRI by inhibiting the activity of the JNK and Caspase-3 signaling pathways, providing new insights and approaches for the treatment of CIRI. However, further research and exploration are needed to elucidate the mechanisms of action and clinical applications of NAC.

Materials and Methods

Middle Cerebral Artery Occlusion (MCAO) Animal Model

A total of 40 C57BL/6 mice were obtained from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). These mice were aged between 6 and 8 weeks and weighed approximately 30 $\pm$ 2 g. Twelve mice were randomly divided into two groups: the Vehicle group and the NAC treatment group. The C57BL/6 mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital at a dosage of 60 mg/kg. The MCAO cell model was established according to the study of Shi *et al.* [24]. The skin was cleaned with an iodine solution, followed by a midline neck incision made with surgical scissors. The common carotid artery and the external carotid artery were identified and ligated with sutures. The internal carotid artery was clamped with vascular forceps. Then, the common carotid artery was incised, and a metal wire was inserted and sutured, followed by ligation. Reperfusion was performed after 2 h. The successful construction of the MCAO model was confirmed by the presence of symptoms such as unstable standing and left-sided hemiplegia in the mice after anesthesia recovery. In the NAC treatment group, mice were intraperitoneally injected with NAC (5 µmol/kg) (C8460, Solarbio, Beijing, China) immediately after the MCAO model was successfully established. After successful modeling, euthanasia was performed by intraperitoneal injection of pentobarbital sodium (100 mg/kg) in mice. The brain specimens were then collected for further analysis. The present study was approved by the Ethics Committee of the Fifth People's Hospital of Jinan (No. 23-ke-21).

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

The mouse brain was rapidly removed, rinsed with cold saline, and placed in a –20 °C refrigerator for 10 min. After the brain tissue became slightly firm, the olfactory bulb, pituitary gland, and lower brainstem were removed, and coronal sections were made from anterior to posterior. The brain was evenly sliced into 2 mm thick sections and divided into five equal parts. These sections were then placed in a 1% TTC solution (G3005, Solarbio, Beijing, China) and incubated at 37 °C in the dark for 30 min, with gently flipping every 5 min. TTC can be reduced to a rose-red color by the dehydrogenase system in normal tissue, resulting in normal tissue staining in rose-red, while ischemic tissue appears white. The infarct area was analyzed using Image J software (version 1.5f, National Institutes of Health, Bethesda, MD, USA).

Hematoxylin and Eosin (HE) Staining

The brain tissue was immersed in 4% paraformaldehyde (P1110, Solarbio, Beijing, China) for fixation for 48 h. Following this, the section from the optic chiasm to the transverse fissure of the brain was extracted and underwent a process of gradient dehydration, paraffin embedding, and consecutive coronal sectioning. The sections were dehydrated using a gradient of xylene and ethanol and then stained with routine HE staining (G1120, Solarbio, Beijing, China) for morphological observation.

mNSS Evaluation

The modified Neurological Severity Score (mNSS) was used to assess the degree of neurological deficits in mice [1]. Neurobehavioral assessments, including motor tests, sensory tests, beam walking tests, and reflex tests, were performed on mice at 1, 3, 7, and 14 days after cerebral ischemia/reperfusion. A higher mNSS score corresponds to a more severe neurological impairment.

Rotarod Test Duration

The rotarod test was used to measure the motor coordination ability of mice [2]. The mice were placed on a 3

Table 1. Primer sequences used in this study.

Primer names (Genes)	Primers sequences
APOE-F	GCTTGGTTTCCCAGGGCTTG
APOE-R	TAGGGCGGGCTGTGCCAGCCCC
CRP-F	AAGTGTCCCAAGTGCAACAA
CRP-R	CGTCTTCCCACATTTCTCG
IL-1β-F	ATGAACTCCTGAACTCAACT
IL-1β-R	TCATCTCGGAGCCTGTAGTG
IL-6-F	GGATGCTACCAAACTGGATA
IL-6-R	CTCTGGCTTTGTCTTTCTTG
TNF-α-F	ATGGGCTCCCTCTCATCAGTTC
TNF-α-R	TCTGCTTGGTGGTTTGCTACGA
CGRP-F	AGTGAAGAAGAAGTTCGCCTGCTG
CGRP-R	CCTCCTGCTCTTCCTCCTGCTC
β-Actin-F	CCCGAGCCGTGTTTCCT
β-Actin-R	GTCCCAGTTGGTGACGATGC

APOE, apolipoprotein E; *CRP*, C-reactive protein; *TNF-α*, tumour necrosis factor-α; *IL*, interleukin; *CGRP*, calcitonin gene-related peptide.

cm diameter non-slip rod rotating at a speed of 10 revolutions per minute. The duration of the mice falling from the rod was recorded.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol (R1100, Solarbio, Beijing, China), treated with RNase-free DNase I, and purified using the RNeasy Mini Kit. The concentration and purity of RNA were determined using a NanoDrop ND-1000, while the integrity of RNA was assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Subsequently, mRNA was reverse transcribed into cDNA using an RT-PCR kit (K16325, MBI, Beijing, China). Amplification was carried out with SYBR Green qPCR Master Mix using cDNA as the template. The amplification process involved an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 min, annealing at 60 °C for 37 s, and extension at 72 °C. The PCR primers were synthesized by Shanghai Shenggong Biotechnology Co., Ltd., Shanghai, China. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences referred to in this chapter can be found in Table 1.

Western Blot

Tissue or cell lysis was carried out on ice using PMSF (10 μL/mL of buffer) for 30 min. The lysates were then centrifuged at 4 °C for 5 min at 11,000 g, and the supernatants were collected. The samples underwent electrophoresis and were subsequently transferred onto PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies against APOE (rabbit anti-mouse, 1:1000, ab51015, Abcam, Cambridge, UK), JNK (rabbit anti-mouse, 1:1000, ab307802, Abcam, Cambridge, UK), c-Jun (rabbit anti-mouse, 1:1000, ab40766, Abcam, Cambridge, UK), p-c-Jun (rabbit anti-mouse, 1:1000, ab32385, Abcam, Cambridge, UK), Cysteine-aspartic Acid Protease-3 (Caspase-3, rabbit anti-mouse, 1:1000, ab32351, Abcam, Cambridge, UK), Cysteine-aspartic Acid Protease-9 (Caspase-9, rabbit anti-mouse, 1:1000, ab202068, Abcam, Cambridge, UK), and GAPDH (rabbit anti-mouse, 1:5000, ab181602, Abcam, Cambridge, UK) after blocking. Subsequently, the membranes were washed with TBST and incubated with secondary antibodies (Anti-Rabbit HRP, Anti-Mouse HRP, 1:1000, ab97051, Abcam, Cambridge, UK) conjugated with horseradish peroxidase. ECL chemiluminescent substrate was added, and the membranes were exposed and imaged. ImageJ software was used for analysis.

Construction of Neuronal Cell Culture and Oxygen-Glucose Deprivation (OGD) Model

Newborn 3-day-old mice (C57BL/6 mice at 6–8 weeks were born after pregnancy) were euthanized through an intraperitoneal injection of pentobarbital sodium (1 mg/mL) (50 mg/kg) following anesthesia. The hippocampus or cortex tissues were dissected and digested with 0.05% trypsin for 30 min and subjected to centrifugation to isolate neuronal cells. Density gradient centrifugation is a standard method that can be employed to isolate different types of cells. In this study, CD56 (ab220360) and CD90 (ab307736) antibodies (Abcam, Cambridge, UK) were used as surface markers for neuronal cells [25]. The labeled cells were analyzed using a flow cytometer (BD FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA). The purified neuronal cells were subsequently plated in a culture dish. After centrifugation, the cells were resuspended in DMEM culture medium supplemented with 5% fetal bovine serum (FBS). The cells were then cultured at a density of 1×10^6 cells/mL for 24 h. Subsequently, the DMEM culture medium (31600, Solarbio, Beijing, China) was replaced with B-27 medium (17504044, Gibco, Beijing, China). Oxygen-glucose deprivation (OGD) was induced after 5 days of culture. The OGD group underwent a gradual transition to a sugar-free culture medium. The glucose concentration was reduced to less than 1 mM, and the cells were incubated in a CO_2 incubator with 95% N_2 and 5% CO_2. The control group cells were cultured in a CO_2 incubator with 5% CO_2. After 4 hours, all cells were switched back to the DMEM culture medium containing 5% fetal bovine serum and cultured in a CO_2 incubator with 5% CO_2. The isolated neuronal cells underwent testing for mycoplasma contamination.

Immunofluorescence

Fixed brain tissues were sliced, dehydrated, permeabilized, embedded in paraffin, and sectioned. The sections were then deparaffinized, rehydrated, and subjected to heat antigen retrieval. After blocking with 5% BSA for 2 h, the sections were incubated overnight at 4 °C with primary

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antibodies against JNK (diluted 1:200, ab307802, Abcam, Cambridge, UK) and Caspase-3 (diluted 1:200, ab32351, Abcam, Cambridge, UK). Following incubation, the sections were warmed for 40 min and washed three times with phosphate-buffered saline (PBS) for 3 min each before being incubated with the fluorescent secondary antibody (Alexa Fluor® 488) (diluted 1:500, ab150077, Abcam, Cambridge, UK) at room temperature in the dark for 2 h, followed by three washes with PBS for 3 min each. After removing PBS, the sections were stained with DAPI for 8 min and then washed three times with PBS in the dark for 3 min each. Finally, an anti-fade mounting medium was applied, and the tissue staining was observed under a fluorescence inverted microscope (IX83, Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling (TUNEL) Staining

The cell samples were fixed to preserve their morphology. This was followed by permeabilization of the cell membrane to allow the entry of dyes into the cells. The TUNEL reaction mixture (T2130, Solarbio, Beijing, China) was prepared, which includes dUTP, TdT, and reaction buffer, and was added to the samples. This step enables TdT to incorporate dUTP at the DNA strand breaks in apoptotic cells. Subsequently, the samples were washed to remove unincorporated dUTP and TdT. Then, fluorescently labeled anti-dUTP antibodies were added to label the incorporated dUTP for observation. Finally, the stained cells were observed under an appropriate microscope (CX53, Olympus, Tokyo, Japan) and image recording and analysis were conducted.

Statistical Analysis

The statistical analysis was performed using SPSS 19.0 (IBM SPSS, Chicago, IL, USA). All data were presented as the mean ± standard deviation (SD). To compare multiple groups, one-way analysis of variance (ANOVA) was employed, followed by Tukey's post hoc test. For comparisons between two groups, *t*-tests were used. A *p*-value less than 0.05 was considered statistically significant.

Results

NAC Improves Neurological Outcomes after MCAO

The modified Neurological Severity Score (mNSS) is a widely used index to assess the degree of neurological deficits in mice after CIRI [26]. The timeline of experimental design for this study is illustrated in Fig. 1. Following the induction of MCAO, the mNSS scores in the NAC group were found to be significantly lower compared to the control group ($p < 0.01$), indicating that NAC treatment can effectively alleviate neurological deficits after CIRI (Fig. 1B). This finding highlights the neuroprotective properties of NAC. In addition, the results of the Rotarod test, which is a commonly used method to evaluate motor coordination and balance in mice, revealed that the NAC group exhibited a longer duration compared to the control group ($p < 0.05$ and $p < 0.01$), indicating that NAC treatment can significantly improve motor coordination and balance in mice after CIRI (Fig. 1C). This suggests that NAC has a positive impact on the recovery of motor function after CIRI.

NAC Treatment Reduces Neuroinflammatory Cytokine Levels after MCAO

In the context of CIRI, the overexpression of certain pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, has been shown to exacerbate inflammatory responses and contribute to neuronal damage [27]. The results presented in Fig. 2A suggest that NAC treatment can significantly reduce the expression levels of TNF-α, indicating that NAC has an inhibitory effect on inflammatory responses. NAC treatment also reduced the expression levels of the inflammatory cytokines IL-1β and IL-6 after CIRI ($p < 0.01$) (Fig. 2B,C). These results suggest that NAC may have a protective effect against CIRI through its inhibitory effect on inflammatory responses. Furthermore, NAC treatment suppressed the mRNA levels of C-reactive protein (*CRP*) and increased the expression levels of calcitonin gene-related peptide (*CGRP*), further highlighting its potential as a therapeutic agent for CIRI ($p < 0.01$) (Fig. 2D,E).

Analysis of APOE Expression after NAC Administration

Apolipoprotein E (APOE) is a lipid transport protein involved in the transport and metabolism of cholesterol and lipids [28]. In CIRI, the expression of APOE may be affected, and its level may be associated with the severity of injury and inflammatory response. Specifically, at 1, 3, and 7 days after CIRI, the expression levels of APOE mRNA in the NAC group were significantly lower than those in the Vehicle group, suggesting an inhibitory effect of NAC on APOE expression ($p < 0.01$) (Fig. 3A–C). This finding was further supported by western blot results, which showed that the expression levels of APOE protein in the NAC group were significantly lower than those in the Vehicle group at 1, 3, and 7 days after CIRI ($p < 0.01$) (Fig. 3D,E).

NAC Reduces Infarct Volume Caused by MCAO

2,3,5-triphenyltetrazolium chloride (TTC) can label viable cells and undamaged tissue in red, leaving the infarcted area unstained and appearing white. The area and volume of cerebral infarction were evaluated using TTC staining. Analysis of Fig. 4A reveals a larger white area in the brain slices of the control group compared to the NAC group, indicating a reduction in the cerebral infarction area with NAC treatment. The volume of cerebral infarction in mice was calculated as a proportion of the hemisphere. The NAC group had a smaller volume of cerebral infarction (*p*

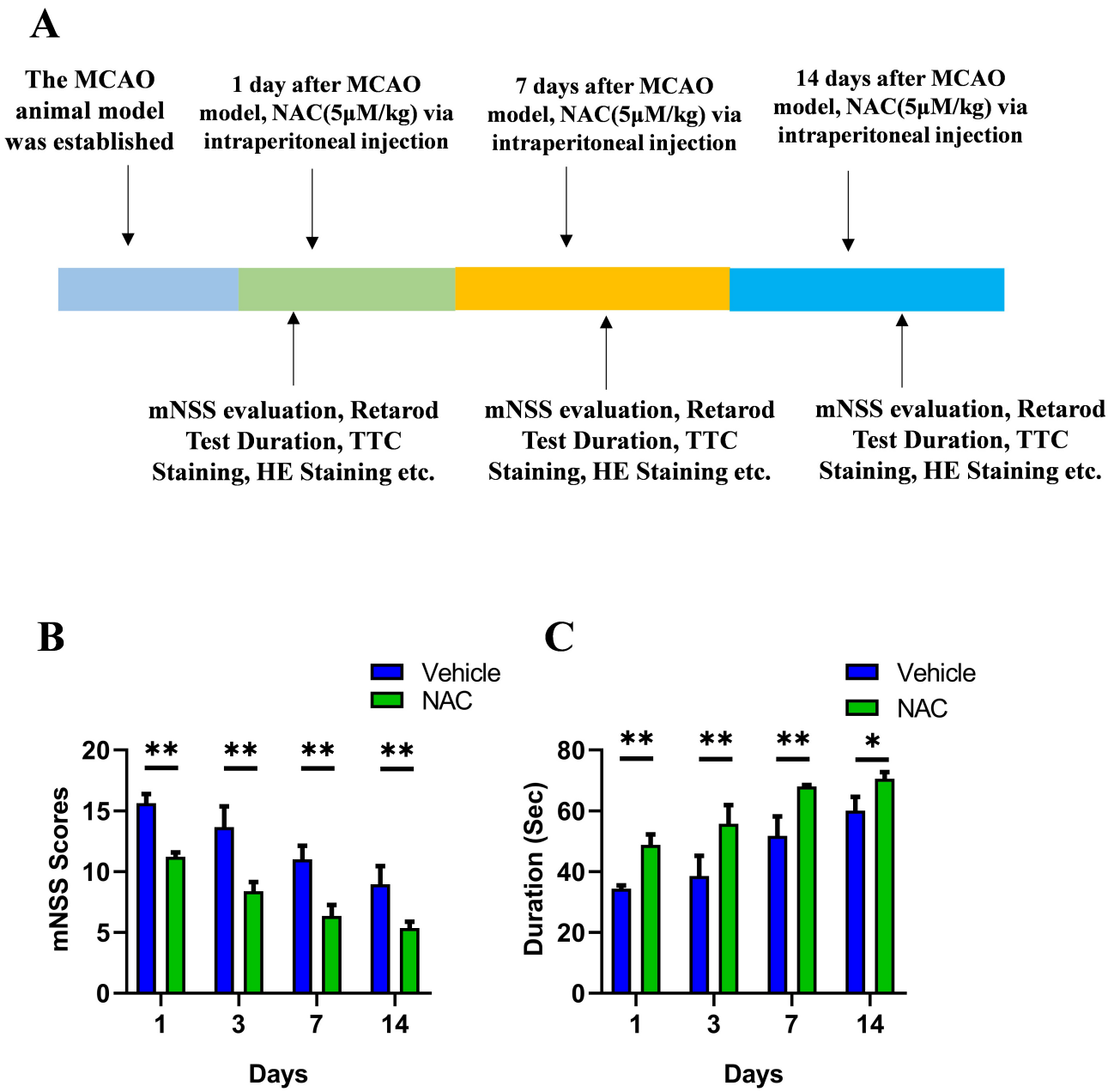

Fig. 1. Neurological outcomes were improved by NAC after t-MCAO. (A) The timeline of experimental design. (B) mNSS assessment in C57BL/6 mice at 1, 3, 7, and 14 days after t-MCAO. (C) Rotarod test duration. n = 5, *$p < 0.05$, **$p < 0.01$. MCAO, Middle Cerebral Artery Occlusion; NAC, N-acetylcysteine; mNSS, modified Neurological Severity Score; TTC, 2,3,5-triphenyltetrazolium chloride.

< 0.01) (Fig. 4B). These findings suggest that NAC administration effectively decreases both the area and volume of cerebral infarction.

HE Staining of Hippocampal CA1 Region in C57BL/6 Mice 3 Days after MCAO

HE staining was used to observe the tissue structure and cell damage in the hippocampal CA1 region of mice. In the brain slices of the control group, the tissue structure of the hippocampal CA1 region appeared disorganized, exhibiting irregular cell arrangement and noticeable cell damage. In contrast, the brain slices from the NAC group displayed a relatively intact tissue structure of the hippocampal CA1 region, characterized by regular cell arrangement and reduced cell damage (Fig. 5A). Quantitative analysis revealed a significantly lower level of tissue structure damage in the NAC group ($p < 0.05$) (Fig. 5B).

NAC Inhibits Cell Apoptosis after OGD

To further investigate the protective effect of NAC on neuronal cells, we established an OGD model. The TUNEL results showed that the hypoxia/reoxygenation group had

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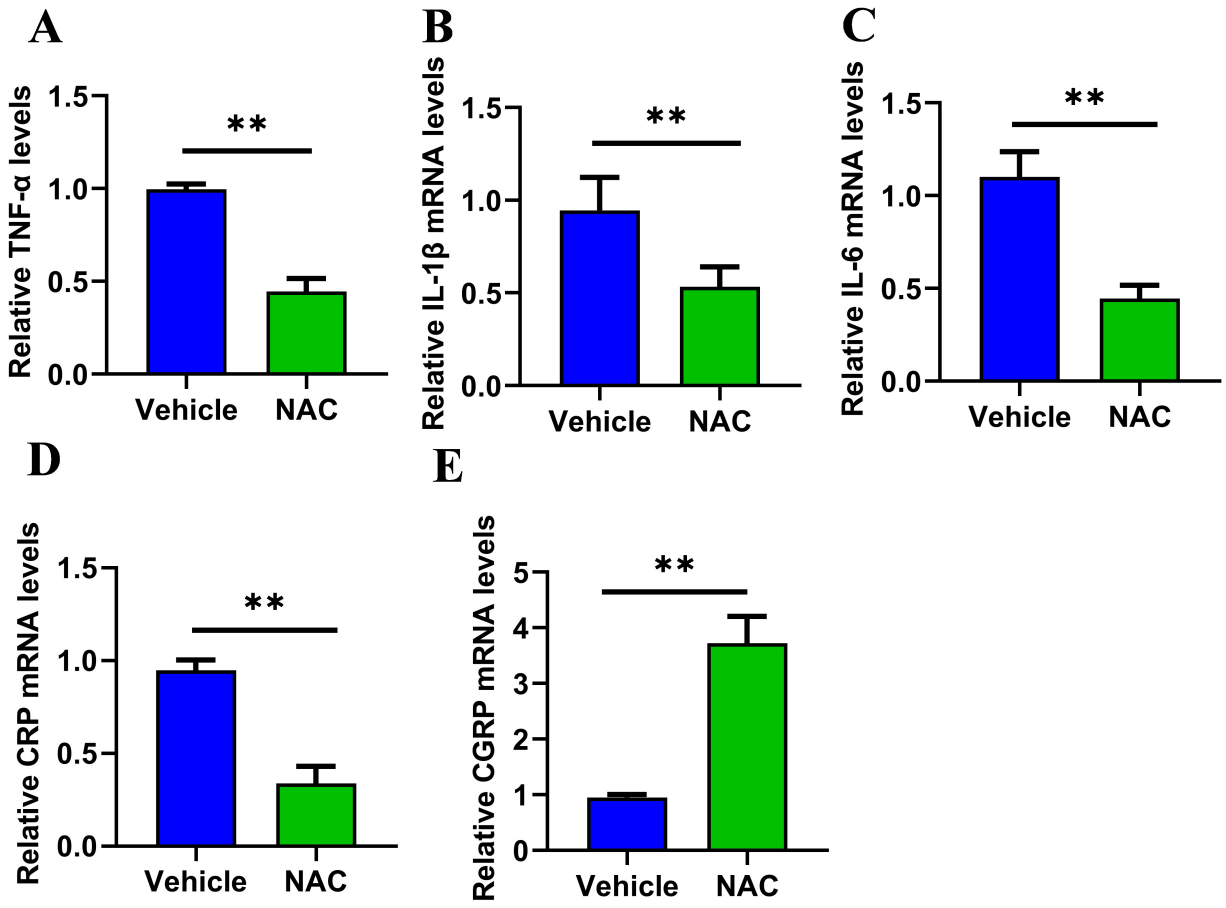

Fig. 2. NAC treatment reduces neuroinflammatory cytokine levels after t-MCAO. (A–E) Expression levels of *TNF-a, IL-1β, IL-6, CRP*, and *CGRP* mRNA. n = 5, $^{**}p < 0.01$.

the highest number of TUNEL-positive cells, indicating a higher rate of cell apoptosis. However, the OGD+NAC group had a significantly lower number of TUNEL-positive cells ($p < 0.01$) (Fig. 6A). JNK is known to regulate cell apoptosis [29], and Caspase-3 is a key executioner enzyme involved in the execution of cell apoptosis [30]. Fig. 6B shows the immunofluorescence staining evaluation of JNK. The staining intensity of JNK was the highest in the OGD group, while it was significantly lower in the OGD+NAC group ($p < 0.01$). The staining intensity of JNK in the control group is intermediate, while the staining intensity of Caspase-3 is the highest in the OGD group and significantly lower in the OGD+NAC group ($p < 0.01$) (Fig. 6C). These results indicate that NAC administration can inhibit cell apoptosis after OGD. NAC may reduce cell apoptosis by inhibiting the JNK signaling pathway and Caspase-3 activity. To further confirm the activating impact of NAC on the JNK/Caspase-3 signaling pathway, we subjected neuronal cells to additional treatments following OGD+NAC administration. This involved the addition of activators for the JNK pathway (Anisomycin) or Caspase-3 (5-Fluorouracil) (Fig. 6D–I). Compared to the control group, OGD treatment markedly increased the protein expression levels of JNK and p-c-Jun. Conversely, OGD+NAC treatment reduced the protein expression of JNK and p-c-Jun compared to the OGD group ($p < 0.05$ and $p < 0.01$). When compared to OGD+NAC, treatment with OGD+NAC+Anisomycin upregulated the protein expression of JNK and p-c-Jun. Notably, these diverse treatments did not influence the protein expression level of c-Jun. Furthermore, OGD treatment significantly elevated the protein expression levels of Caspase-3 and Caspase-9 compared to the control group. In contrast, OGD+NAC treatment diminished the protein expression of Caspase-3 and Caspase-9 compared to the OGD group. In comparison to OGD+NAC, the treatment involving OGD+NAC+5-Fu significantly increased the protein expression levels of Caspase-3 and Caspase-9 ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Flow cytometry analysis indicated positive expression of CD56 (97.9%), and CD90 (94.1%) in isolated neuronal cells, as depicted in Fig. 6J.

Discussion

In this study, we investigated the potential mechanisms through which NAC attenuates CIRI by suppressing the JNK/Caspase-3 signaling pathway. Our results indicate

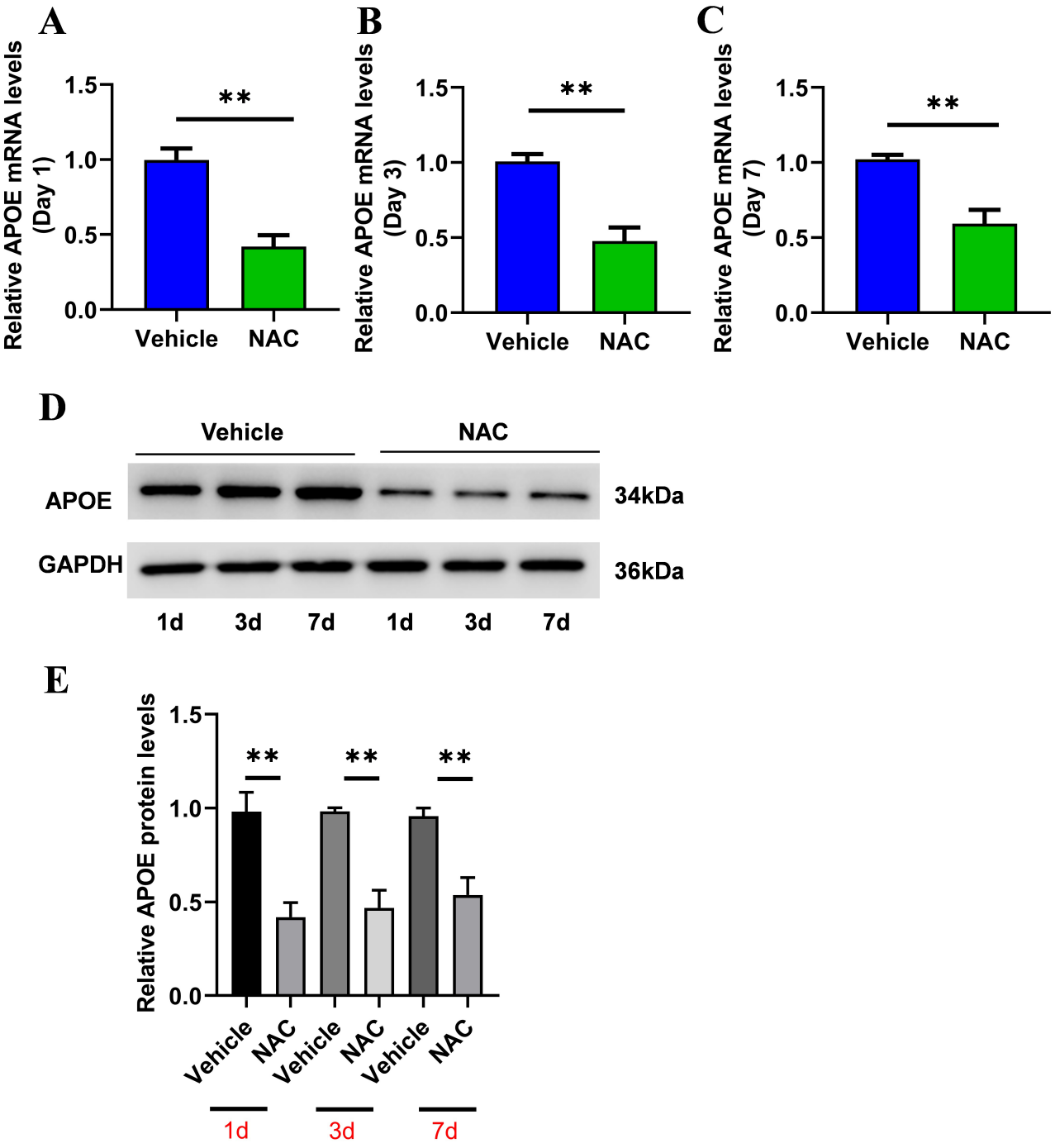

Fig. 3. Analysis of APOE expression after NAC administration. (A) Expression levels of *APOE* mRNA at 1 day after t-MCAO. (B) Expression levels of *APOE* mRNA at 3 days after t-MCAO. (C) Expression levels of *APOE* mRNA at 7 days after t-MCAO. (D,E) Expression levels of APOE protein after t-MCAO. n = 5, $^{**}p < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

that NAC has significant neuroprotective effects, which may be attributed to its multiple actions, including antioxidant, anti-inflammatory, and anti-apoptotic properties.

Our experimental results suggest that NAC has the potential to enhance neurological outcomes following MCAO, indicating its neuroprotective effects and its potential as a therapeutic agent for promoting recovery and repair of the nervous system. These findings are consistent with previous studies, further supporting that NAC may serve as an effective therapeutic agent for CIRI [31]. The neuroprotective effects of NAC may be attributed to its antioxidant properties, as oxidative stress is a key pathological mechanism in CIRI [31]. Specifically, research has shown that the post-ischemic administration of NAC in experimental stroke rat models can protect the brain against free radical damage, cell apoptosis, and inflammation [32]. Fur-

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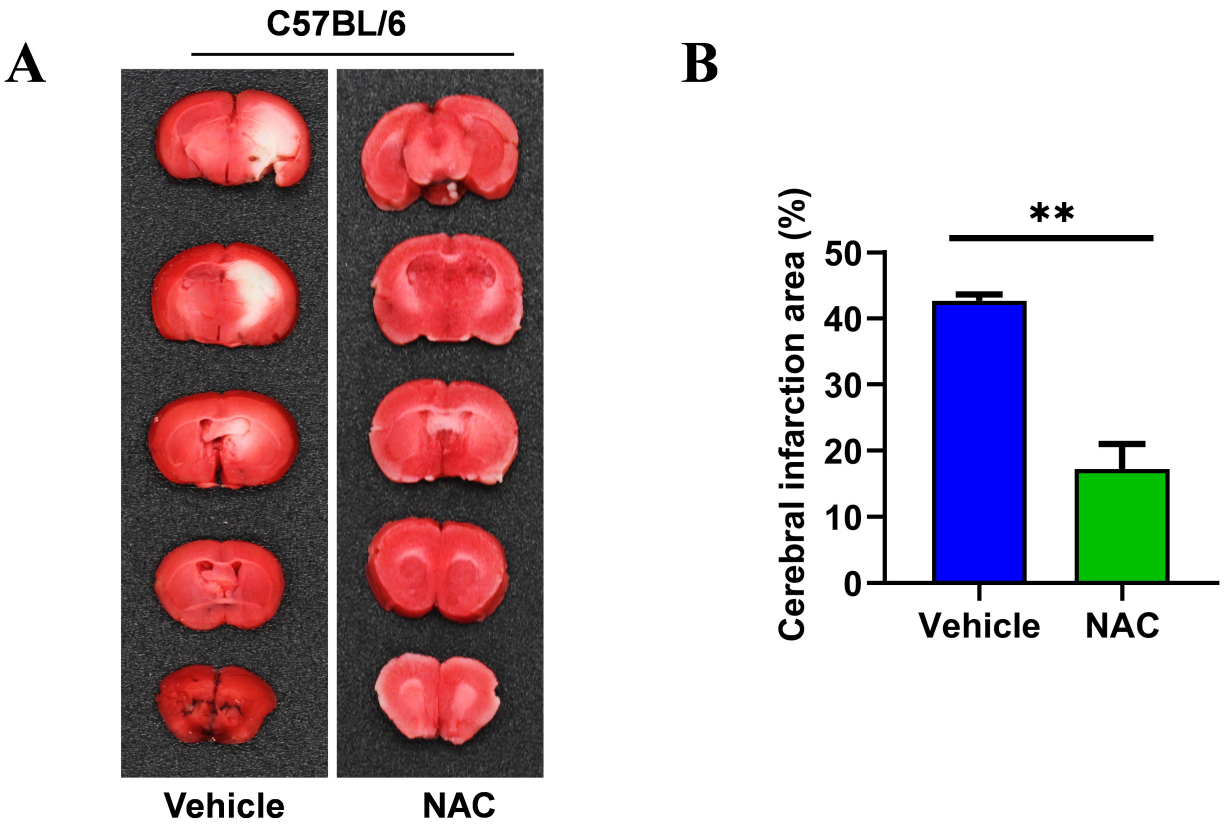

Fig. 4. NAC reduces infarct volume caused by t-MCAO. (A) Representative images of brain slices stained with TTC in different groups. (B) Calculation of mouse brain infarct volume as a percentage of the hemisphere. n = 5, $^{**}p < 0.01$.

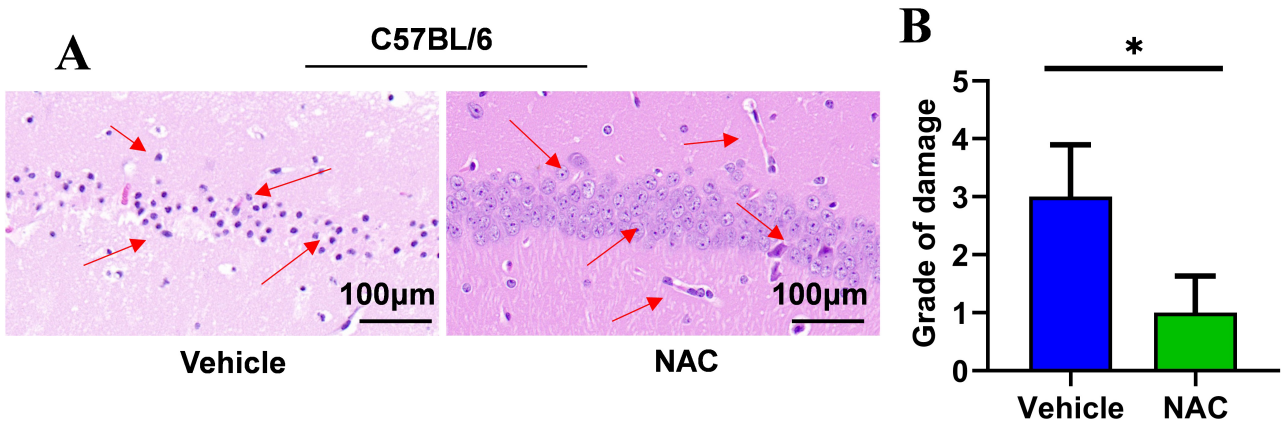

Fig. 5. HE-stained hippocampal CA1 region slices of C57BL/6 mice at 3 days after t-MCAO. (A) HE staining results of brain tissue regions. Red arrows represent tissue structure, cell arrangement, and areas of cell damage. (B) Quantitative analysis showing the level of inflammatory damage. n = 5, $^{*}p < 0.05$. HE, hematoxylin and eosin.

thermore, our experimental results demonstrate that NAC significantly reduces the levels of neuroinflammatory factors after MCAO. Neuroinflammation plays a crucial role in CIRI, and excessive activation of neuroinflammatory responses can lead to neuronal damage and death [33,34]. Moreover, a report indicates that NAC, as an effective antioxidant, can reduce short-term axonal injury and hippocampal neuronal loss, demonstrating neuroprotective effects [35]. The anti-inflammatory effects of NAC may be achieved through the inhibition of inflammatory mediator release and modulation of the inflammatory response, thereby alleviating CIRI. This finding is consistent with the known anti-inflammatory effects of NAC. Additionally, NAC decreases the expression of APOE, which is a significant factor in the development of CIRI. NAC may promote neural repair and regeneration, as well as alleviate injury,

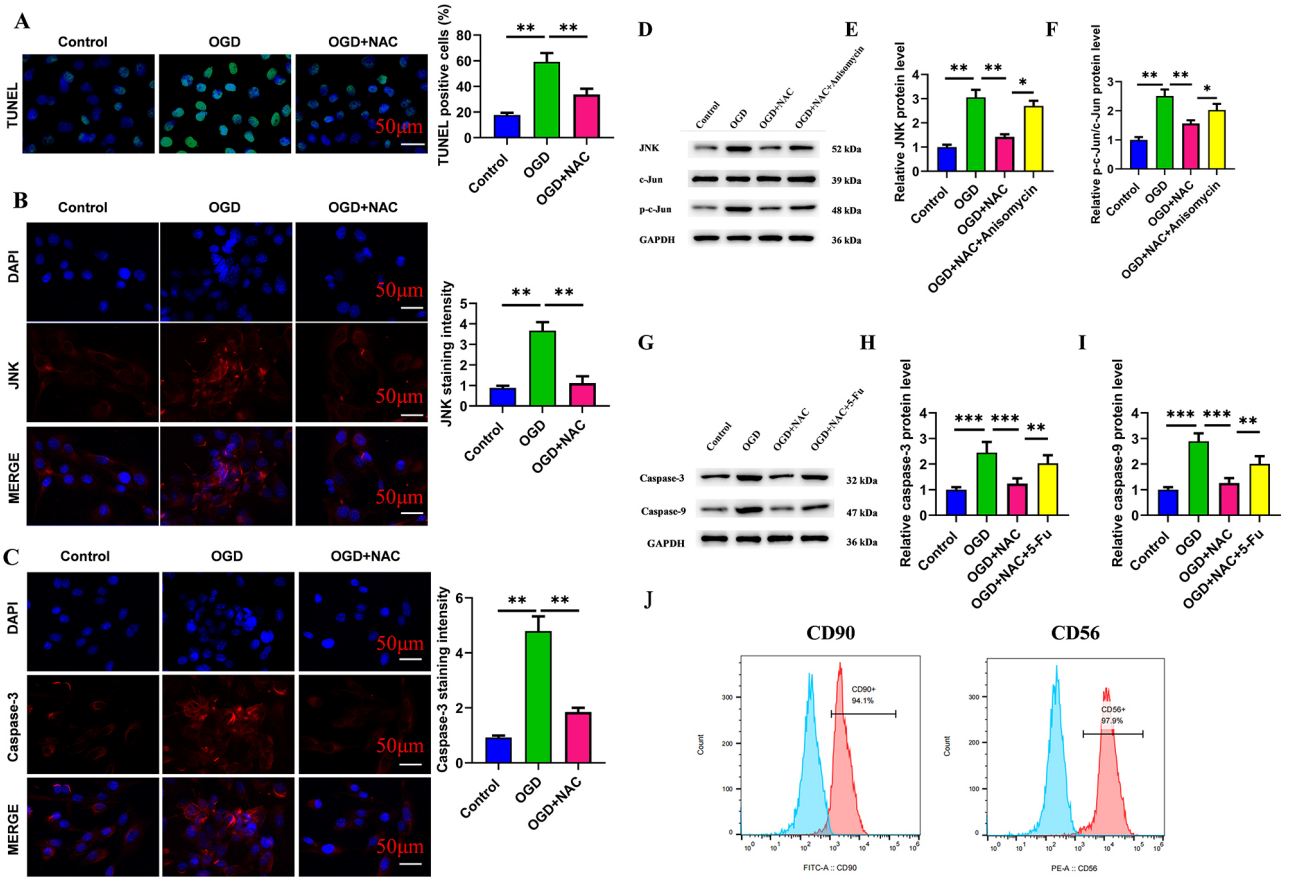

Fig. 6. NAC inhibits cell apoptosis after OGD. (A) Representative images of TUNEL-positive cells. Scale bar: 50 μm. (B) Immunofluorescence staining evaluating changes in c-Jun N-terminal Kinase (JNK) expression. Scale bar: 50 μm. (C) Immunofluorescence staining evaluating changes in Cysteine-aspartic Acid Protease-3 (Caspase-3) expression. Scale bar: 50 μm. (D–I) The protein expression levels of JNK, c-Jun, p-c-Jun, Caspase-3 and Cysteine-aspartic Acid Protease-9 (Caspase-9) were determined by western blot. (J) Expression levels of the surface markers CD56 and CD90 on neuronal cells. n = 6, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. OGD, oxygen-glucose deprivation.

by regulating the expression of APOE. Infarct volume is a crucial indicator for assessing the severity of CIRI. The size of the infarct volume is directly related to the extent of the damage. NAC has been found to reduce infarct volume, demonstrating its efficacy in attenuating CIRI.

The JNK signaling pathway is a key regulatory mechanism involved in stress response and cellular apoptosis [36,37]. In CIRI, hypoxia and reperfusion injury can activate the JNK signaling pathway, leading to inflammation and cellular apoptosis, thereby exacerbating the extent of brain damage [38]. A recent study suggests that NAC can protect testicular tissue from ischemia-reperfusion injury by inhibiting endoplasmic reticulum stress (ER) and apoptosis. Additionally, cell apoptosis pathways associated with endoplasmic reticulum stress may play a crucial role in testicular injury [19].

Moreover, the JNK signaling pathway can influence the production and clearance of free radicals through the regulation of intracellular oxidative stress levels, further aggravating brain injury. Caspase-3, a pivotal execution enzyme in cellular apoptosis, exhibits increased activity, leading to cellular apoptosis. During CIRI, hypoxia and reperfusion injury can activate Caspase-3, resulting in neuronal apoptosis and further exacerbating brain injury. Furthermore, Caspase-3 can influence cell survival by regulating the expression of apoptosis-related proteins such as Bcl-2 and Bax, further contributing to brain injury [39]. Therefore, the JNK and Caspase-3 signaling pathways play crucial roles in CIRI. The study by Liu *et al.* [40] indicated that Bexarotene can alleviate focal cerebral ischemia-reperfusion injury by suppressing the JNK/Caspase-3 signaling pathway. The activation of these pathways can lead to inflammation and cellular apoptosis, thereby exacerbating brain injury. Conversely, inhibiting the activity of these two signaling pathways can alleviate inflammation and cellular apoptosis, thereby protecting neurons from damage and reducing the extent of brain injury.

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and Homeostatic Agents

While NAC treatment has demonstrated efficacy in the mouse MCAO model, its potential effectiveness in humans could be limited by the time window. Additionally, the mouse model may not comprehensively reflect the complexity of human diseases. Significant differences in drug metabolism between mice and humans may result in treatment effects observed in the mouse model not directly translating to humans.

We found that NAC can effectively inhibit cellular apoptosis after MCAO. Further investigation revealed that NAC may exert this protective effect by suppressing the JNK/Caspase-3 signaling pathway. Specifically, NAC may inhibit the activation of JNK and the expression of Caspase-3, thereby suppressing cellular apoptosis and alleviating CIRI.

Conclusions

Our findings support the hypothesis that NAC alleviates CIRI by inhibiting the JNK/Caspase-3 signaling pathway, providing new theoretical evidence for the application of NAC in the treatment of CIRI. However, our study has some limitations, including its focus on cellular and animal models. Furthermore, the specific mechanisms of action of NAC require further investigation. In future studies, we aim to explore the mechanisms of action of NAC and validate our findings in larger sample sizes.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

WG designed the research study. XNJ performed the research. YL provided help and advice on the western blot experiments. FZ analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The present study was approved by the Ethics Committee of the Fifth People's Hospital of Jinan (No. 23-ke-21).

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Conflict of Interest

The authors declare no conflict of interest.

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