Rhoifolin attenuates damage to hippocampal neuronal culture model of acquired epilepsy *in vitro* by regulating NF-κB/iNOS/COX-2 axis

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ORIGINAL ARTICLE

Abstract

To assess the effect of Rhoifolin (ROF [apigenin 7-O-enneohesperidoside]) on the damage to hippocampal neuronal culture model of acquired epilepsy (AE) and investigate its possible mechanisms. A hippocampal neuronal culture model of AE was established through incubating HT-22 cells with $MgCl_2$ free medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to assess the effect of ROF on cell viability and apoptosis exposed to epilepsy. The oxidative stress and secretion of inflammatory cytokines were measured by reverse transcription-quantitative polymerase chain reaction and enzyme-linked-immunosorbent serologic assay, respectively. Immunoblot assays were performed to determine the protein expression levels of nuclear factor kappa B/nitric oxide synthases/cyclooxygenase-2 (NF-κB/iNOS/COX-2) axis. ROF increases viability and reduces apoptosis of AE medium-treated HT-22 cell line. ROF relieves oxidative stress in AE medium-treated HT-22 cell line. ROF decreases the levels of pro-inflammatory cytokines in AE medium-treated HT-22 cell line. The functional effects of ROF on AE medium-treated HT-22 cell line is through inhibiting NF-κB/iNOS/COX-2 axis. ROF increased viability, decreased apoptosis, suppressed oxidative stress, and reduced pro-inflammatory cytokine levels in an epilepsy model *in vitro* by inhibiting NF-κB/iNOS/COX-2 axis. ROF might serve as a potential drug for epilepsy treatment.

Keywords: epilepsy; rhoifolin (ROF, apigenin 7-O-β-neohesperidoside); neuronal activity; apoptosis; NF-κB/iNOS/COX-2 axis

Introduction

Epilepsy, a chronic brain disorder characterized by seizures, is caused or triggered by a persistent tendency of neuronal overstimulation and hypersynchronization (Torii *et al.*, 2022). The latest statistics indicate that around 65 million people worldwide suffer from epilepsy. A patient's life is severely affected by autonomous loss of activity (d'Orio *et al.*, 2022). Despite the existence of multiple anti-epileptic drugs, one-third of the patients do not respond to these drugs, which only control the clinical manifestations of epilepsy and do not affect the occurrence or pathology of epilepsy (Cui and Zhang, 2022). Oxidative stress, neuronal apoptosis, and inflammation are primary causes of epilepsy (Beltran-Corbellini *et al.*, 2022). During epileptic seizures, significant levels of free radicals and accumulation of pro-inflammatory cytokines have been analyzed in the brain, which are key

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factors for the recurrence of epilepsy and are related to the severity of its pathology.

Rhoifolin (ROF [apigenin 7-O-β-neohesperidoside]), a flavonoid glycoside that belongs to apigenin family (Coussio, 1964), contains multiple components. It can be isolated from different plants such as sumac, artichokes, tomatoes, bananas, and grapes. In addition, large amounts of ROF have been found in various citrus plants (Aoki *et al.*, 2017; Yasue *et al.*, 1967). ROF also serves as a drug of choice in different types of diseases. The antioxidant and anti-inflammatory properties of ROF were found in a variety of diseases, including osteoarthritis, diabetes, hepatitis, and pneumonia (Peng *et al.*, 2020; Xiong *et al.*, 2021; Yan *et al.*, 2021).

The role of ROF in different diseases has been widely studied (Negm *et al.*, 2022). For example, ROF attenuated osteoclast-stimulated osteolysis by inhibiting mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) axis. ROF regulated oxidative stress and pro-inflammatory cytokine levels by the suppression of NF-κB pathway (Liao *et al.*, 2019). ROF could also ameliorate titanium particle-stimulated osteolysis by targeting NF-κB and MAPK pathways (Fang *et al.*, 2020). Inhibitory effects of ROF on nitric oxide synthases (iNOS) and C–C motif chemokine ligand 2 (CCL2) expression were also investigated in lipopolysaccharide (LPS)-induced RAW264.7 cells. ROF inhibits acetylcholinesterase (AChE) activity and modulates cholinergic activity to improve scopolamine-induced anxiety, amnesia, and oxidative stress in zebrafish (Chen *et al.*, 2022). However, whether ROF could alleviate hippocampal neuron damage caused by epilepsy has not been reported.

In this study, we assess the effects of ROF on hippocampal neuron damage caused by epilepsy and investigate the underlying mechanisms. An epilepsy model was established by incubating HT-22 cells with magnesium chloride ($MgCl_2$)-free medium. The results demonstrated that ROF increased viability, decreased apoptosis, suppressed oxidative stress, and reduced levels of pro-inflammatory cytokines in hippocampal neuronal culture model of acquired epilepsy (AE) by inhibiting NF-κB/iNOS/cyclooxygenase-2 (COX-2) axis.

Materials and methods

Cell culture

HT-22 neurons, an immortalized hippocampal neuronal cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 5% CO_2 at 37°C.

Treatment

Cells were divided into the following groups: control, AE, AE+ROF (5, 10, and 20 uM), and AE+JSH-23. For induction of AE, HT-22 cells were incubated with $MgCl_2$ free medium containing 145-mM NaCl, 2.5-mM KCl, 10-mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-mM $CaCl_2$, 10-mM glucose, and 0.002-mM glycine, at pH 7.3, accompanied with osmolarity that was adjusted to 325 mOsm by sucrose (AE medium). ROF was dissolved in dimethyl sulfoxide (DMSO), and the control group was treated with an equal volume of DMSO. ROF was administrated at the doses of 5, 10, and 20 μM for 24 h. JSH-23 (25 μM), an inhibitor of NF-κB transcriptional activity, was μ added into cells for 24 h.

Cell viability

HT-22 cells were plated at a density of 3×10^3 cells/well into 96-well plates. After indicated treatment, the viability of cells in different groups was assessed after the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were incubated for another 4 h before measuring optical density (OD) at a wavelength of 490 nm.

TUNEL Staining

After indicated treatment, cells were fixed in formaldehyde, rinsed in tris-buffered saline (TBS) and then stained with cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The sections were measured using microscope (Olympus, Tokyo, Japan). The apoptotic cells were counted manually.

Measurement of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase (MPO) levels

After indicated treatment, cells were collected for measuring levels of MDA, SOD, GSH, and MPO using commercial kits (Jiancheng Bioengineering Institute of Nanjing, Jiangsu Province, China). Cells were homogenized and centrifuged (1,000 g) for 20 min and the supernatant was collected. Then the samples were added, shaken gently, mixed, and covered for reaction at 37°C for 2 h. A microplate reader was used to detect the OD

value of each well at a wavelength of 450 nm. The experiment was repeated thrice.

Real-time polymerase chain reaction (RT-PCR)

Cellular total RNA was extracted by TRIzol reagents (Thermo, Rockford, USA). Total RNA was reversely transcribed into complementary DNA (cDNA) using Moloney Murine leukemia virus (M-MLV) reverse transcriptase (Promega Corporation, Madison, WI, USA). The cDNA was amplified using the following primers: tumor necrosis factor-α (TNF-a): GGTGCCTATGTCTCAGCCTCTT and GCCATAGAACTGATGAGAGGGAG; interleukin (IL)-1β: ACAAGGAGAAGAAAGTAATGAC and GCTGTAGAGTGGGCTTAT; IL-6: AGACAGCCACTCACC and TTC TGCCAGTGCCTCTT; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): AGAAGGCTGGGGCTCATTTG and AGGGGCCATCCACAGTCTTC.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as described by Liao *et al.* (2019). After indicated stimulations, cell supernatants were subjected to ELISA (ELISA kit, Shanghai Xitang Biotechnology Co. Ltd., Shanghai, China) to determine the levels of TNF-α, IL-6, and IL-1β following the manufacturer's guidelines.

Western blotting assay

Western blotting assay was performed as described by Chen *et al.* (2022). Proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The samples were collected and electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferring on polyvinylidene difluoride (PVDF) membranes, and blocked with 5% fat-free

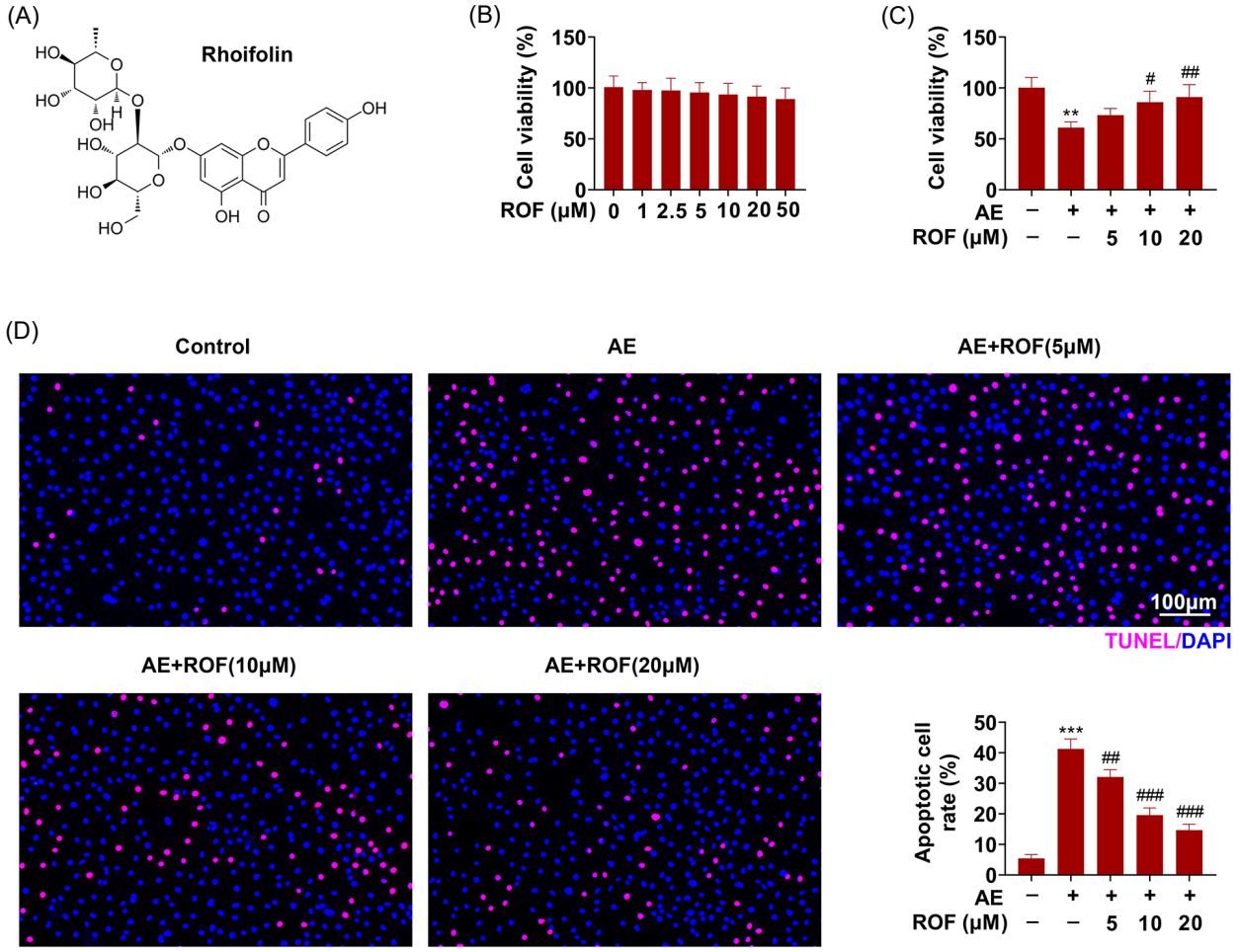

Figure 1. ROF increases viability and reduces apoptosis of AE medium-treated HT-22 cell line. (A) Molecular formula of ROF. (B) Viability of HT-22 cell line in response to various concentrations of ROF, as detected by MTT assay. (C) Viability of HT-22 cell line in response to AE and various concentrations of ROF, as detected by MTT assay. (D) Apoptosis of HT-22 cell line in response to AE and various concentrations of ROF, as detected by TUNEL assay. **$P < 0.01$ and ***$P < 0.001$ vs. control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. AE group. All experiments were performed in three replicates.

milk. Subsequently, membranes were incubated with primary antibodies targeting p-p65 (mouse, 1:1,000; Abcam Inc., Cambridge, UK), p65 (mouse, 1:1,000; Abcam), p-IκBα (mouse, 1:1,000; Abcam), IκBα (mouse, 1:1,000; Abcam), iNOS (mouse, 1:1,000; Abcam), COX-2 (mouse, 1:1,000; Abcam), and β-actin (mouse, 1:1,000; Abcam) for 1 h. Finally, the membranes were conjugated with anti-mouse Immunoglobulin G (IgG; Abcam) for 1 h. Specific proteins were visualized with enhanced chemiluminescence detection kit (ECL, Thermo Fisher Scientific, Rockford, USA).

Statistics

GraphPad 6.0 was used for statistical analysis. Three replicates were performed for each experiment. One-way ANOVA and Student's *t*-test were used for statistical comparisons. $P < 0.05$ was considered statistically significant.

Results

ROF increases viability and reduces apoptosis of AE medium-treated HT-22 cell line

MTT assay was performed to evaluate the viability of HT-22 cell line after exposed to ROF. HT-22 cell line was incubated with different concentrations of ROF. The molecular formula of ROF is shown in Figure 1a. Cell viability was minimally affected after exposure to ROF (Figure 1b). Then the cells were exposed to AE medium and ROF, and subjected to MTT assay. AE treatment led to reduction in the viability of HT-22 cell line. ROF treatment improved the decreased cell viability stimulated by AE (Figure 1c). Moreover, the apoptosis of HT-22 cell line was enhanced by AE treatment, while ROF treatment reversed this phenomenon as determined by the TUNEL assay (Figure 1d). Collectively, ROF increased viability and reduced apoptosis of AE medium-treated HT-22 cell line.

ROF relieves oxidative stress in AE medium-treated HT-22 cell line

Owing to the important role of oxidative stress in AE, the levels of SOD, MDA, GSH, and MPO were analyzed in different groups. The results demonstrated that the levels of MDA and MPO were increased, and that of SOD and GSH were decreased in the AE group. Treatment of ROF reversed the levels of SOD, MDA, GSH, and MPO in a dose-dependent manner (Figure 2). These results suggested that ROF reduced oxidative stress in AE medium-treated HT-22 cell line.

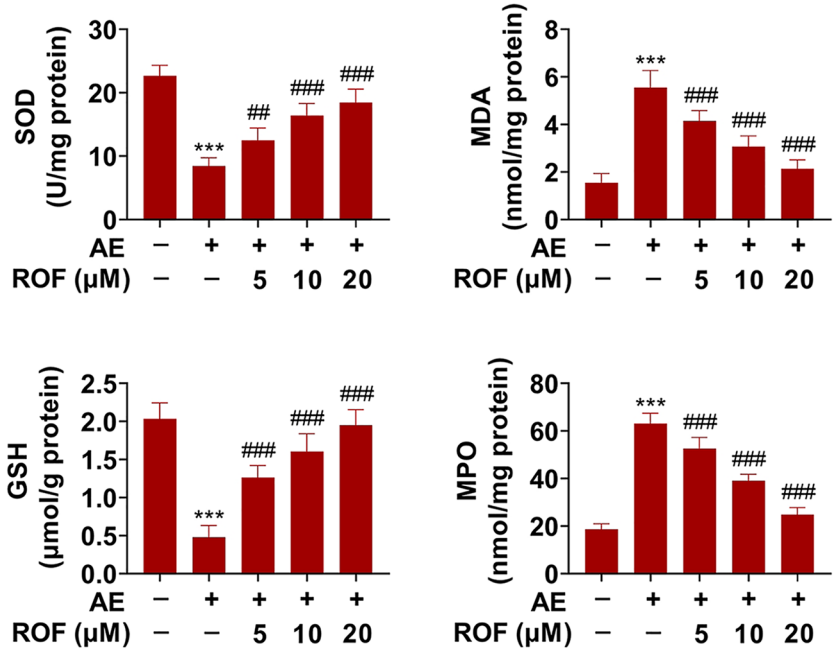

Figure 2. ROF relieves oxidative stress in AE medium-treated HT-22 cell line. The levels of SOD, MDA, GSH, and MPO in response to AE and various concentrations of ROF. ***$P < 0.001$ vs. control group. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ vs. AE group. All experiments were performed in three replicates.

ROF decreases the levels of pro-inflammatory cytokines in AE medium-treated HT-22 cell line

Inflammatory responses in HT-22 cell line were assessed by determining messenger RNA (mRNA) expressions and levels of pro-inflammatory cytokines through RT-quantitative(q)PCR and ELISA, respectively. Stimulation of AE medium induced inflammation as evidenced by increased mRNA expressions and levels of TNF-a, IL-1b, and IL-6 (Figures 3a and b). However, ROF treatment relieved the levels of TNF-a, IL-1b, and IL-6 in HT-22 cell line. Therefore, ROF reduces the levels of pro-inflammatory cytokine in AE medium-treated HT-22 cell line.

Functional effects of ROF on AE medium-treated HT-22 cell line is through inhibiting NF-κB/iNOS/COX-2 axis

In order to reveal the possible mechanisms underlying the functional effects of ROF on the viability and apoptosis, oxidative stress, and inflammatory response in AE medium-treated HT-22 cell line, the expression levels of NF-κB/iNOS/COX-2 pathway were detected. It was observed that the protein levels of p-p65, p-Ikb, iNOX, and COX-2 were elevated in AE medium-treated HT-22 cell line, while the same were decreased by ROF, but dramatically declined by JSH-23 treatment (Figure 4a). Consistently, JSH-23 treatment aggravated the protective roles of ROF on the viability and apoptosis in AE medium-treated HT-22 cell line (Figures 4b–d). Besides, JSH-23 treatment aggravated the protective roles of ROF on the oxidative stress in AE medium-treated HT-22 cell line (Figure 4e). Therefore, these results indicated that the functional effect of ROF on AE medium-treated HT-22 cell line was by inhibiting NF-κB/iNOS/COX-2 axis.

Discussion

Epilepsy is a chronic disease of sudden abnormal discharge of brain neurons, resulting in temporary brain dysfunction (Xu *et al.*, 2022). The annual prevalence of active epilepsy with seizures is 4.6% (Wei *et al.*, 2022). Globally, there are 5–6-million active epilepsy patients, with about 400,000 new epilepsy patients added annually (Liang *et al.*, 2022). Epilepsy has become the second most common neurological disease. However, no effective anti-epileptic drug has been initiated till now. Therefore, development of effective treatment for epilepsy is crucial. In this study, the results revealed that ROF could attenuate damage caused to hippocampal neuronal culture model of AE, established by incubating HT-22 cell line with AE medium.

RhoifolinROF increased the viability, decreased the apoptosis, suppressed oxidative stress, and reduced levels of pro-inflammatory cytokines levels in AE medium-treated

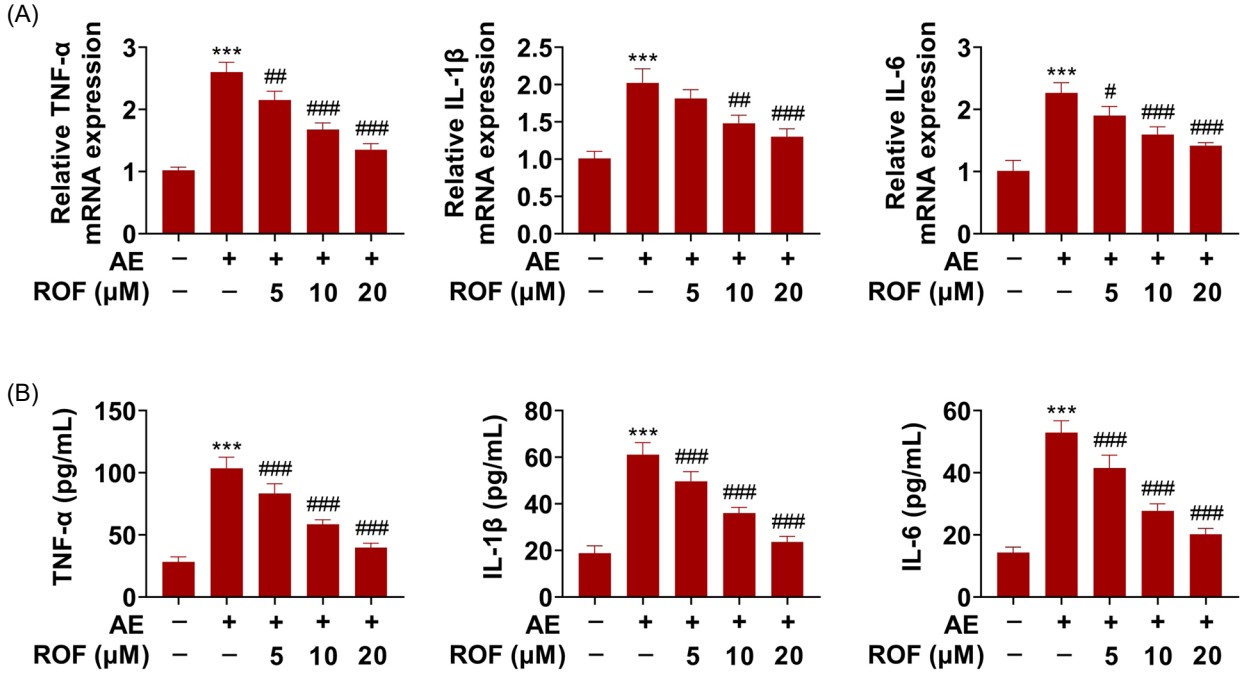

Figure 3. ROF decreases the levels of pro-inflammatory cytokines in AE medium-treated HT-22 cell line. (A and B) The mRNA expressions and levels of TNF-α, IL-1b, and IL-6 were determined by RT-qPCR and ELISA, respectively. ***$P < 0.001$ vs. control group, #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ vs. AE group. All experiments were performed in three replicates.

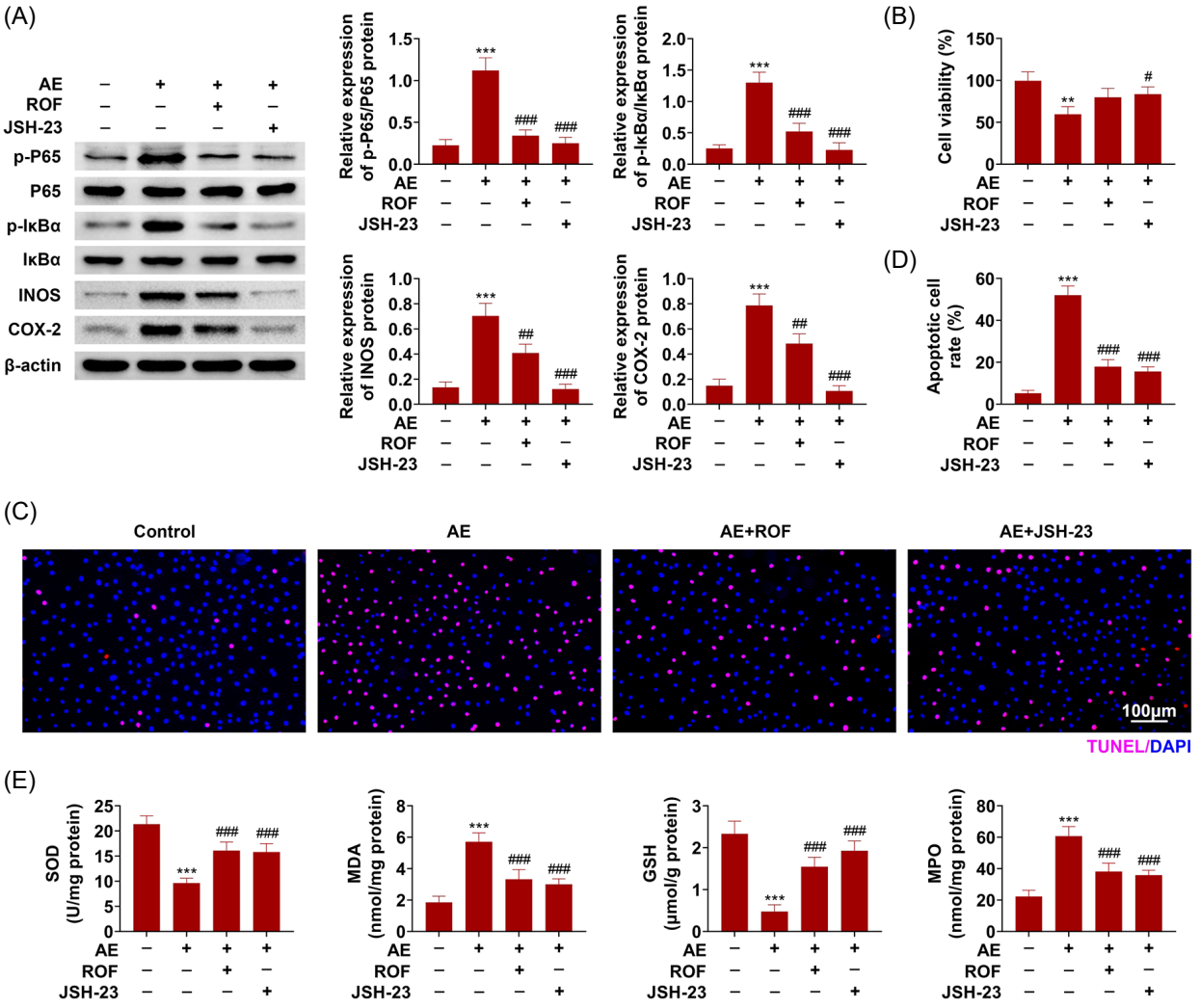

Figure 4. The functional effects of ROF on AE medium-treated HT-22 cell line are through inhibiting NF-κB/iNOS/COX-2 axis. (A) Immunoblot assay depicted the expression levels of p-p65, p-IkB, iNOX, and COX-2 in HT-22 cell line after co-treatment of AE and ROF or JSH-23. (B) The viability of HT-22 cell line after co-treatment of AE and ROF or JSH-23. (C and D) The apoptosis of HT-22 cell line after co-treatment of AE and ROF or JSH-23. (E) Levels of SOD, MDA, GSH, and MPO in HT-22 cell line after co-treatment of AE and ROF or JSH-23. **$P < 0.01$ and ***$P < 0.001$ vs. control group. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ vs. AE group. All experiments were performed in three replicates.

HT-22 cell line. ROF is thus established to have the ability to attenuate the damage caused to hippocampal neurons and could serve as a promising drug for epilepsy. Consistently, the diverse biological activities of ROF have been revealed in different diseases (Brinza *et al.*, 2020; Chen *et al.*, 2022). It exerts antioxidant and anti-inflammatory properties in many diseases such as osteoarthritis, diabetes, hepatitis, pneumonia, etc. (Peng *et al.*, 2020). ROF also inhibited AChE activity and modulated cholinergic activity to improve anxiety and oxidative stress in zebrafish.

Rhoifolin could alleviate inflammation in animal models of acute inflammation via inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ)/NF-κB pathway. Similarly, ROF was also found to affect inflammation via NF-κB pathway in AE medium-treated HT-22 cell line. In addition, ROF regulated oxidative stress and pro-inflammatory cytokine levels in rheumatoid arthritis via NF-κB suppression. Similarly, ROF could also ameliorate titanium particle-stimulated osteolysis as well as osteoclastogenesis via receptor activator of nuclear factor kappa-B ligand (RANKL)-induced NF-κB pathway. These studies confirmed strong connection between ROF and NF-κB pathway.

NF-κB pathway plays an important role in immune and stress-related processes. NF-κB is present in almost all cell types (Li *et al.*, 2020). It plays a fundamental role in regulating immune response to infection. Improper

regulation of NF-κB has been associated with inflammatory and autoimmune diseases (He *et al.*, 2019). NF-κB is also involved in synaptic plasticity and memory processes (Koosha *et al.*, 2019). The results of this study revealed that ROF could attenuate damage to hippocampal neuronal culture model of AE via NF-κB pathway. However, the precise mechanism needs additional investigations.

Conclusion

In conclusion, this study intended to assess the effect of ROF on hippocampal neuron damage caused by epilepsy and investigate its possible mechanisms. The results demonstrated that ROF increased viability, decreased apoptosis, suppressed oxidative stress, and reduced levels of pro-inflammatory cytokines in hippocampal neuronal culture model of AE by inhibiting NF-κB/iNOS/COX-2 axis.

Competing interests

The authors stated that there were no conflicts of interest to disclose.

Ethics approval

This article did not contain any experiment carried out by any of the authors with human or animal participants.

Author Contribution

Huizhen Qi and Liang Liu designed and carried out the experiments. Both authors analyzed and interpreted the data and prepared the final manuscript.

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