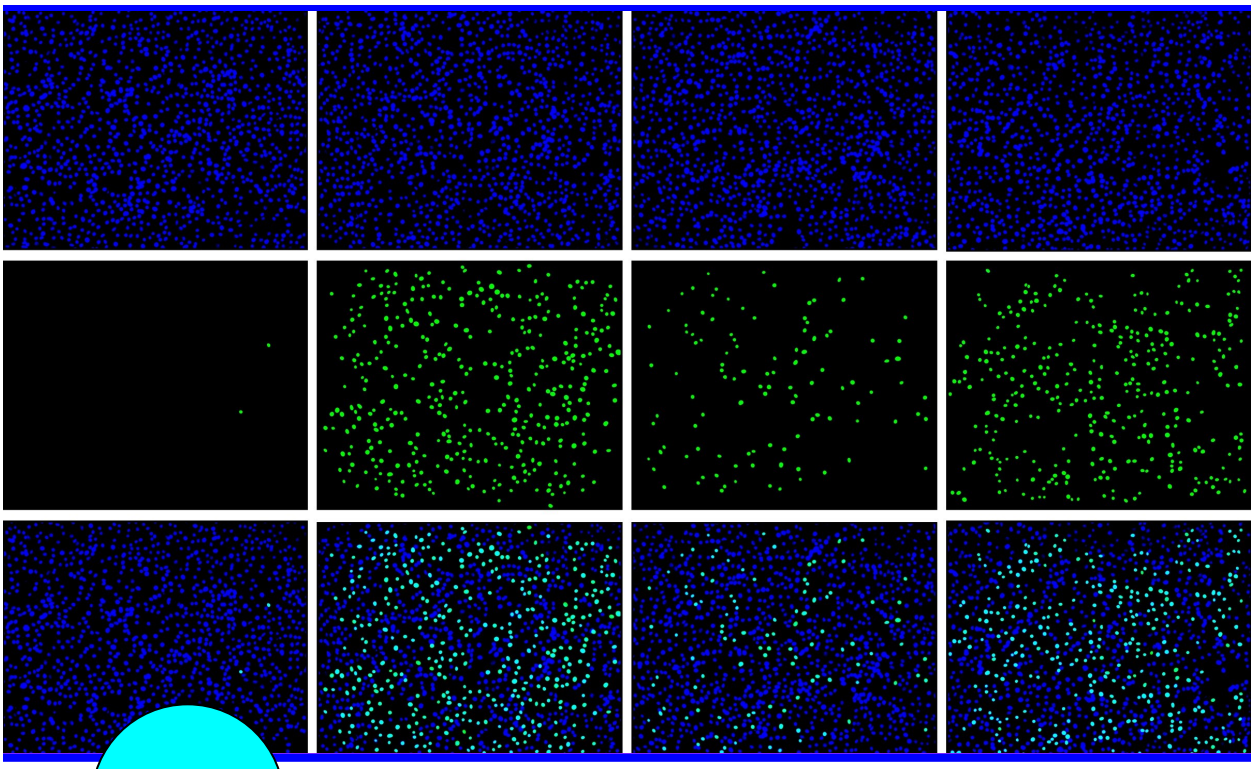

BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info; www.bdpsjournal.org
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2022; 17: 23-31
ISSN: 1991-0088

Sesquiterpene compound α-cyperone relieves the injury in neurons undergoing oxygen-glucose deprivation/reoxygenation

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Article Info

Received: 11 February 2022
Accepted: 10 May 2022
Available Online: 6 June 2022
DOI: 10.3329/bjp.v17i2.58188

Cite this article:
Wang J, Gao XF. Sesquiterpene compound α-cyperone relieves the injury in neurons undergoing oxygen-glucose deprivation/reoxygenation. Bangladesh J Pharmacol. 2022; 17: 23-31.

Abstract

The present study aimed to explore the effects of α-cyperone, an extract of *Cyperus rotundus*, on PC12 cells, as well as the underlying mechanism. Following the cells were induced by oxygen-glucose deprivation/reoxygenation (OGD/R), the viability, morphology, inflammation, oxidative stress and apoptotic levels in the cells were evaluated. To explore the mechanism of α-cyperone, cells were treated with 3-TYP, a sirtuin-3 (SIRT3) inhibitor, and then the effects of 3-TYP on the function of α-cyperone were assessed. α-Cyperone was found to reduce OGD/R-induced damage to neuronal viability and alleviate inflammation, oxidative stress, and apoptosis. In addition, α-cyperone could elevate SIRT3 and decline acetyl-forkhead box O1 (FOXO1) levels, and 3-TYP broke the effects of α-cyperone on the aforementioned aspects in the PC12 cells. In conclusion, α-cyperone activated SIRT3 and FOXO1 deacetylation, and alleviated OGD/R-induced cell inflammation, oxidative stress, and apoptosis.

Introduction

As the acceleration of population aging, cerebrovascular diseases have seriously affected human health. Stroke is one of the major factors in cerebrovascular diseases that trigger human death and permanent disability (Akinyemi et al., 2019). Stroke is divided into hemorrhagic stroke and ischemic stroke (Paul and Candelario-Jalil, 2021). Therein, ischemic stroke caused by cerebral artery stenosis or blockage that severely reduces cerebral circulation blood volume accounts for 80-87% (Black et al., 2015).

Currently, the use of intravenous thrombolytic drugs and intravascular interventional therapy to restore patency of occluded blood vessels and increase blood flow is the primary measure for clinical treatment of ischemic stroke (Silva and Nogueira, 2020). Nevertheless, reperfusion will trigger a series of complex pathophysiological processes, such as the release of excitatory neurotransmitters, calcium overload, the release of inflammatory factors, and the activation of apoptotic pathways, which can eventually lead to further damage to the ischemic brain tissue, namely ischemia/reperfusion (I/R) injury (Orellana-Urzúa et al., 2020; Wang et al., 2020c; You et al., 2018).

At present, the pathophysiological mechanism of cerebral I/R injury is not fully elucidated. Therefore, exploring the mechanism of cerebral I/R injury and the development of novel effective drugs for the treatment of cerebral I/R injury is still an urgent problem to be solved.

Cyperus rotundus L., a Chinese herbal medicine widely distributed in tropical and subtropical, belongs to the Cyperaceae family (Rocha et al., 2020). *C. rotundus* has efficacy in the treatment of inflammation, pyrosis, gastrointestinal and metabolic disorders (Kanagali et al., 2021). It has been extensively applied in clinical

treatments in some countries (Kamala et al., 2018a; Lu et al., 2021). Notably, the research of scientific researchers on *C. rotundus* never ceased. Traditional medicine contains multi-target characteristics, which drives more pharmacological experiments to be carried out (Kamala et al., 2018b). For example, *C. rotundus* could alleviate cerebral ischemic damage and memory dysfunction in rats (Dabaghian et al., 2015). The hydroalcoholic extract of *C. rotundus* reduced the expression of the Bcl-x1 anti-apoptotic gene in the hippocampus of rats following global I/R injury (Farahani and Hashemi, 2016). The oligomeric flavonoids in *C. rotundus* could improve the neurological deficit and excitotoxicity of rats with cerebral I/R injury (Sunil et al., 2011). Contemporary medicine tends to explore the pharmacology of single structural compounds rather than mixtures. It is known that the components of *C. rotundus* include volatile oil, sugars, flavonoids, triterpenoids, alkaloids, anthraquinones, etc. (Hu et al., 2017; Kamala et al., 2018a) The sesquiterpene compound α-cyperone, which is present in the volatile oil, has been analyzed for its specific compound structure (Figure 1A), and it has been proved to be the active ingredient of the *C. rotundus* (Zhang et al., 2021).

Rat pheochromocytoma PC12 has the general characteristics of neuroendocrine cells and is broadly used in neurophysiological research (Wang et al., 2020b). Hence, PC12 cells were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) to mimic I/R *in vitro* (Sun et al., 2018). The present study aimed to explore the effects of α-cyperone on PC12 cells undergoing OGD/R, as well as the underlying mechanism.

Materials and Methods

Cell culture and treatment

PC12 cells were purchased from Cobioer Co. Ltd. (China) and cultured in DMEM (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2.

To simulate I/R *in vitro*, the PC12 cells (2x10^5 cells/well) were subjected to OGD/R. Briefly, the cells were incubated in a glucose-free, serum-free DMEM medium in an incubator containing 95% N_2 and 5% CO_2 at 37°C for 2 hours. Subsequently, α-cyperone (0-30 μM; MedChemExpress, China) (Huang et al., 2018) or 3-TYP (sirtuin-3 (SIRT3) inhibitor; 50 μM; MedChemExpress) (Zeng et al., 2019) was added to the cells followed by a normal incubation with 5% CO_2 at 37°C for a further 24 hours (Wang et al., 2021). The cells that have not been treated with α-cyperone were used as the OGD/R group. The cells cultured with DMEM containing 10% FBS for the full 24 hours period at 5% CO_2 atmosphere were used as the control group. Cell morphology was observed under a microscope (magnification, x200; Olympus Corporation).

Cell counting kit 8 (CCK-8) assay

Cell viability was determined using a CCK-8 kit (Beyotime, China) (Huang, 2018). Briefly, PC12 cells (5x10^3 cells/well) were seeded in 96-well plates and processed as aforementioned. Then 10 μL CCK-8 reagent was added to each well and cells were given a further 2 hours incubation. The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, LLC).

Determination of reactive oxygen species (ROS) and superoxide dismutase (SOD)

The levels of ROS and SOD in PC12 cells were separately determined using specific assay kits (Beyotime) according to the manufacturer's protocols. The OD value was measured using a microplate reader.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from PC12 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific). RT-qPCR was performed using the BeyoFast™ SYBR Green One-Step RT-qPCR kit (Beyotime) according to the manufacturer's protocols. The procedure was as follows: reverse transcription at 50°C for 15 min; predenaturation at 95°C for 2 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec; and fusion at 95°C for 15 sec. GAPDH was used as an internal reference and data were analyzed using the comparative Ct method (Schmittgen and Livak, 2008). The primer sequences (5′→3′) are as follows: TNF-α, forward, TTCCCAAATGGGCTCCCTCT, reverse, GTGGGCTACGGGCTTGTCAC; IL-1β, forward, TCCAGGATGAGGACCCAAGC, reverse, TCGTCATCATCCCACGAGTCA; IL-6, forward, TCTGGGAAATCGTGGAAATGAG, reverse, TCTCTGAAGGACTCTGGCTTTGTC; IL-10, forward, CTGGCTCAGCACTGCTATGT, reverse, GCAGTTATTGTCACCCCGGA; GAPDH, forward, CTCTCTGCTCCTCCCTGTTC, reverse, TACGGCCAAATCCGTTCACA.

TUNEL assay

PC12 cells (2 x 10^4 cells/well) were seeded into a 24-well plate and the assay was performed using a TUNEL kit (C1086; Beyotime) according to the manufacturer's protocols. Briefly, cells were fixed with 4% paraformaldehyde and subsequently permeated with PBS containing 0.3% Triton X-100. After blocking with 3% H_2O_2 for 5 min, TUNEL staining was performed and then DAPI was used to counterstain the nuclei for 10 min. The results were photographed under a microscope (magnification, x200).

Statistical analysis

All data are presented as mean ± standard deviation (SD) and experiments were performed in triplicate.

Box 1: Western Blotting

Principle

Western blotting is used for detection and characterization of target proteins. It is based on the principle of immuno-chromatography where proteins are separated into polyacryl-amide gel according to their molecular weight.

Requirements

Primary antibodies against catalase (GTX110704; 1:1,000; GeneTex), manganese superoxide dismutase (MnSOD) (GTX116093; 1:5,000; GeneTex), SIRT3 (GTX33499; 1:1,000; GeneTex), FOXO1 (GTX50499; 1:1,000; GeneTex), acetyl-FOXO1 (PA5-104560; 1:1,000; Thermo Fisher Scientific), Bcl-2 (ab32124; 1:1,000; Abcam), Bax (ab32503; 1:1,000; Abcam), cleaved PARP (ab32064; 1:10,000; Abcam) or GAPDH (ab8245; 1:1,000; Abcam); HRP-conjugated goat anti-rabbit secondary antibodies (ab181602; 1:10,000; Abcam); RIPA lysis buffer (Solarbio, China); SDS-PAGE (10% gel); PVDF membranes (Millipore, Merck)

Procedure

Step 1: Total proteins were extracted from PC12 cells using RIPA lysis buffer (Solarbio, China) on ice.

Step 2: Following the quantification of proteins with the BCA method, SDS-PAGE (10% gel) was performed, and proteins were transferred onto PVDF membranes.

Step 3: The membranes were blocked in 5% non-fat milk for 2 hours at room temperature, and incubated with primary antibodies against catalase, manganese superoxide dismutase, SIRT3, FOXO1, acetyl-FOXO1, Bcl-2, Bax, cleaved PARP or GAPDH at 4°C overnight.

Step 4: Afterward, they were incubated with HRP-conjugated goat anti-rabbit secondary antibodies for another 2 hours at room temperature.

Step 5: Enhanced chemiluminescence reagent (Thermo Fisher Scientific) was used to visualize the protein bands and the gray values were analyzed using ImageJ version 1.8 software.

Reference

Cheng et al., 2019

References (Video)

Eslami and Lujan, 2010; Lim et al., 2021; Shen et al., 2021

Statistical analysis was performed using a one-way ANOVA followed by Tukey's *post hoc* test (Hazra and Gogtay, 2016) in GraphPad Prism version 8.0 software (GraphPad Software). $P<0.05$ indicated a statistically significant difference.

Results

Effects on neuronal viability and inflammation

The effect of gradient concentrations of α-cyperone on PC12 cell viability was determined using a CCK-8 assay. The result revealed that α-cyperone at the concentration of 0 to 30 μM had no damage to cell viability (Figure 1B). After the cells were induced by OGD/R, the CCK8 assay was still used to detect cell viability, and the cell morphology was observed concurrently. It was found that OGD/R significantly reduced the cell viability, and the cell morphology was rounded under the microscope. However, the viability of cells treated with α-cyperone was significantly higher than that of the OGD/R group, and the cell morphology tended to be normal as the concentration of α-cyperone increased (Figure 1C-D). In addition, the levels of inflammatory factors in the cells were detected. The results of RT-qPCR analysis indicated that TNF-α, IL-1β, and IL-6 levels were significantly elevated in cells induced by OGD/R, and α-cyperone treatment reduced their levels. Meanwhile, the level of IL-10 was decreased in cells induced by OGD/R, and treatment with α-cyperone increased its level (Figure 1E).

Effects on neuronal oxidative stress and apoptosis

Then the effect of α-cyperone on cell oxidative stress was investigated. The level of ROS and SOD in each group of cells was determined using assay kits. The ROS level was increased in the OGD/R group, and α-cyperone treatment declined the level in cells (Figure 2A). Whereas the SOD level was decreased in the OGD/R group, and α-cyperone treatment elevated its level in cells (Figure 2B). Moreover, the levels of catalase and MnSOD were determined using Western blotting. The expression levels of these two proteins were significantly decreased in the OGD/R induction group, and significantly up-regulated in the α-cyperone -treated cells (Figure 2C). Subsequently, the effect of α-cyperone on the level of cell apoptosis was evaluated using TUNEL assay and Western blotting. The results of the TUNEL staining displayed that the number of apoptotic cells increased after OGD/R induction, and the apoptotic rate of the α-cyperone treatment group was partially relieved (Figure 2D-E). In the Western blotting, the protein expression levels of Bcl2, Bax, and cleaved PARP were examined. The level of Bcl2 was decreased significantly after OGD/R induction and increased with α-cyperone concentration-dependently. The alteration trend of Bax and cleaved PARP levels was just the opposite of that of Bcl2 (Figure 2F).

Effects on SIRT3/FOXO1 signaling and 3-TYP

The expression levels of SIRT3, acetyl-FOXO1, and FOXO1 were determined using Western blotting. SIRT3 and FOXO1 protein expression levels were declined significantly in the cells undergoing OGD/R induction and increased as the concentration of α-cyperone elevated. While the level of acetyl-FOXO1 was increased in the OGD/R induction group, yet it was down-regulated in the α-cyperone treatment groups (Figure 3A). Afterward, 3-TYP was applied to treat cells

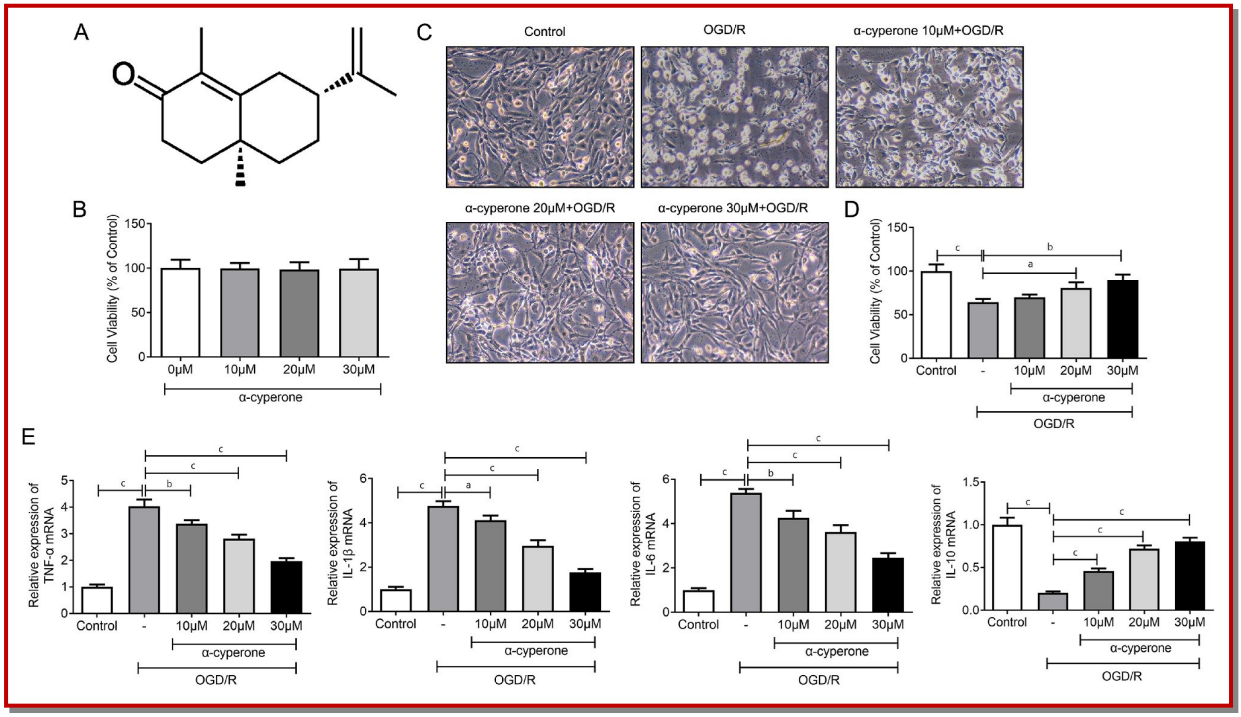

Figure 1: α-Cyperone reduces OGD/R-induced damage to neuronal viability and alleviates inflammation. The chemical structure of α-cyperone (A). The effect of gradient concentrations of α-cyperon on PC12 cell viability was determined using a CCK-8 assay (B). Following the cells were induced by OGD/R, cell viability and cell morphology were assessed (C-D). The levels of inflammatory factors in the cells were determined using RT-qPCR analysis (E). $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$

along with α-cyperone. The expression levels of SIRT3, acetyl-FOXO1, and FOXO1 in the cells co-treated with α -cyperone and 3-TYP were determined using Western blotting. In cells subjected to OGD/R stimulation with α-cyperone and 3-TYP treatment, compared with cells not treated with 3-TYP, the levels of SIRT3 and FOXO1 were decreased, and that of acetyl-FOXO1 was increased (Figure 3B). Next, cell viability and morphology in the 3-TYP treatment group were evaluated. The cells in the 3-TYP treatment group became rounded and their viability decreased (Figure 3C-D). Moreover, TNF-α, IL -1β, and IL-6 levels were significantly elevated in cells treated with 3-TYP, whereas that of IL-10 was decreased (Figure 3E). These results indicated that α-cyperone influenced neuronal viability and inflammation via regulating SIRT3/FOXO1 signaling.

Effects on 3-TYP on neuronal oxidative stress and apoptosis

The ROS and SOD levels in the cells treated with 3-TYP were likewise determined. Compared with the cells that did not receive 3-TYP treatment, the level of ROS in the 3-TYP treatment group was increased, and that of SOD was declined (Figure 4A-B). The protein expression level of catalase and MnSOD in the 3-TYP treatment group was also affected, manifesting a downward trend (Figure 4C). Furthermore, the result of the TUNEL assay revealed that 3-TYP treatment increased the rate of apoptotic cells (Figure 4D-E). And from the results of Western blotting, 3-TYP treatment declined the protein expression level of Bcl2 and elevated that of Bax and cleaved PARP (Figure 4F). These results suggested that α-cyperone also influenced neuronal oxidative stress and apoptosis via regulating SIRT3/ FOXO1 signaling.

Discussion

Although significant progress has been obtained in the prevention and acute treatment of stroke, the current options for the treatment of ischemic stroke are still limited, and they can only be used within a short period after symptoms appear (Herpich and Rincon, 2020). Therefore, it is necessary to develop more effective treatment methods and drugs to improve the living standards of patients. Studies have shown that cerebral I/R injury may be the result of a combination of factors such as oxidative stress, cell apoptosis, energy metabolism disorders, excitatory amino acid toxicity, and cell necrosis (Maida et al., 2020). In this article, we explored the role of α-cyperone in neuroinflammation, oxidative stress, and apoptosis. First of all, our experimental results revealed that α-cyperone with the highest experimental concentration of 30 μM did not harm the viability of PC12 cells, and could reduce the damage to cell viability following the cells were stimulated by OGD/R.

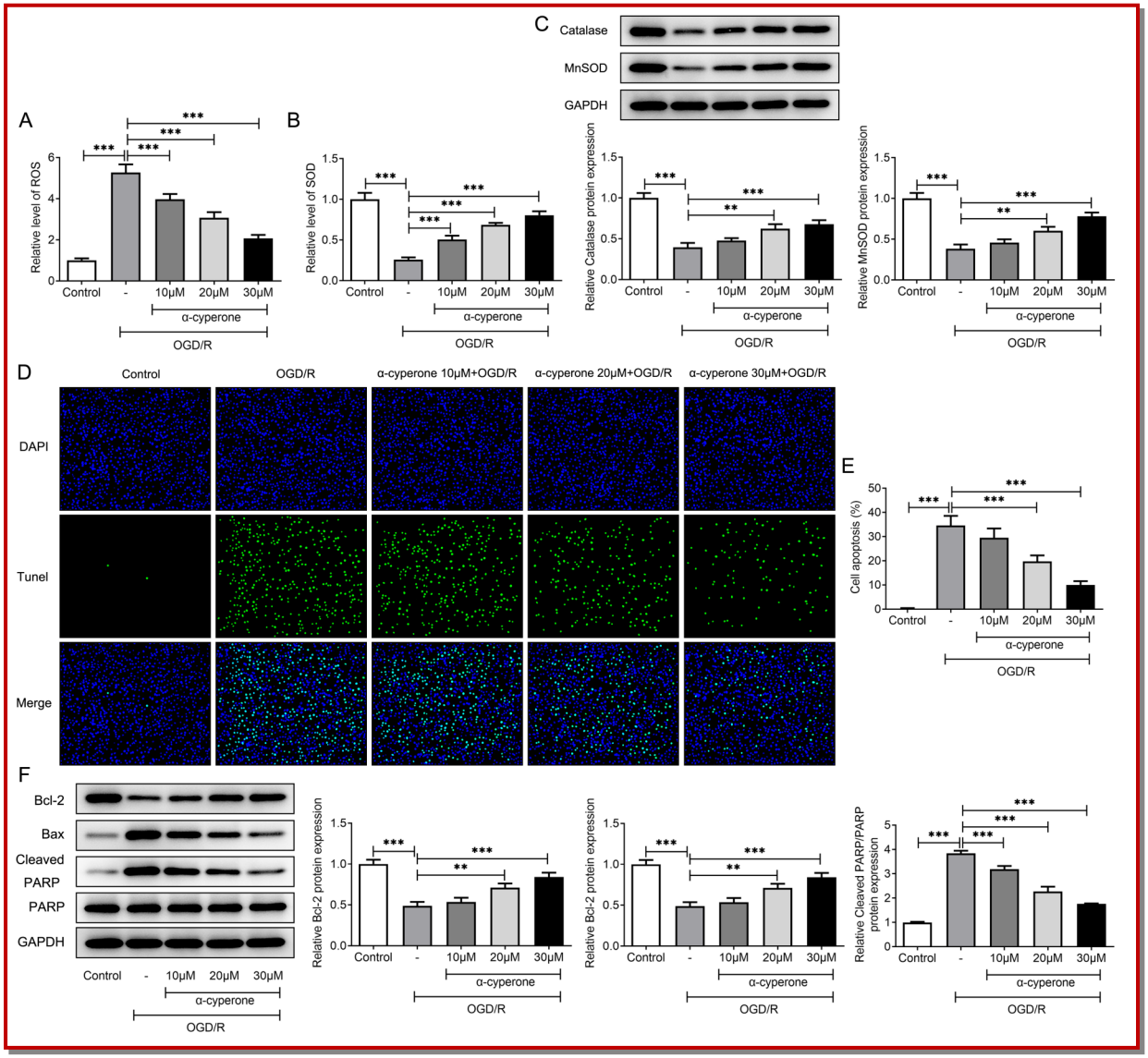

Figure 2: α-Cyperon reduces OGD/R-induced neuronal oxidative stress and apoptosis. The level of ROS (A) and SOD (B) in each group of cells was determined using assay kits. The levels of catalase and MnSOD were determined using Western blotting (C). The effect of α-cyperone on the level of cell apoptosis was evaluated using TUNEL assay (D-E) and Western blotting (F). Magnification, x200. [a]p<0.05, [b]p<0.01, [c]p<0.001

Moreover, the ischemic stroke will up-regulate the expression of inflammatory factors, leading to an increase in the permeability of the blood-brain barrier (BBB) (Chi et al., 2020). Hence, we studied the effect of α-cyperone on the level of inflammatory factors and found that α-cyperone could also effectively reduce the release of inflammatory factors in cells, which suggested that α-cyperone might be capable to reduce the inflammatory damage caused by I/R. Previous studies have shown that α-cyperone can inhibit the activation of microglia induced by tumor-derived DNA, inhibit the activation of microglia NF-κB, and reduce the neuroinflammatory response (Gao et al., 2021). These results indicate that α-cyperone has the potential to alleviate neuroinflammation resulting from different causes.

During reperfusion, although the restoration of oxygen can restore organ functions, oxygen participates in the formation of oxygen free radicals and has a toxic effect on nerve cells, causing neuronal restoration homeostasis, which in turn leads to further tissue damage and neuronal apoptosis (He et al., 2020). The endogenous apoptotic pathway mediated by mitochondria is the major and vital apoptotic pathway (Datta et al., 2020). Therefore, we continued to evaluate the effects of α-cyperone on the levels of oxidative stress and apoptosis after cells undergoing OGD/R. The experimental results displayed that α-cyperone significantly reduced the level of intracellular ROS, increases the levels of SOD, catalase, and MnSOD. And α-cyperone significantly reduced the rate of cell apoptosis, presenting a

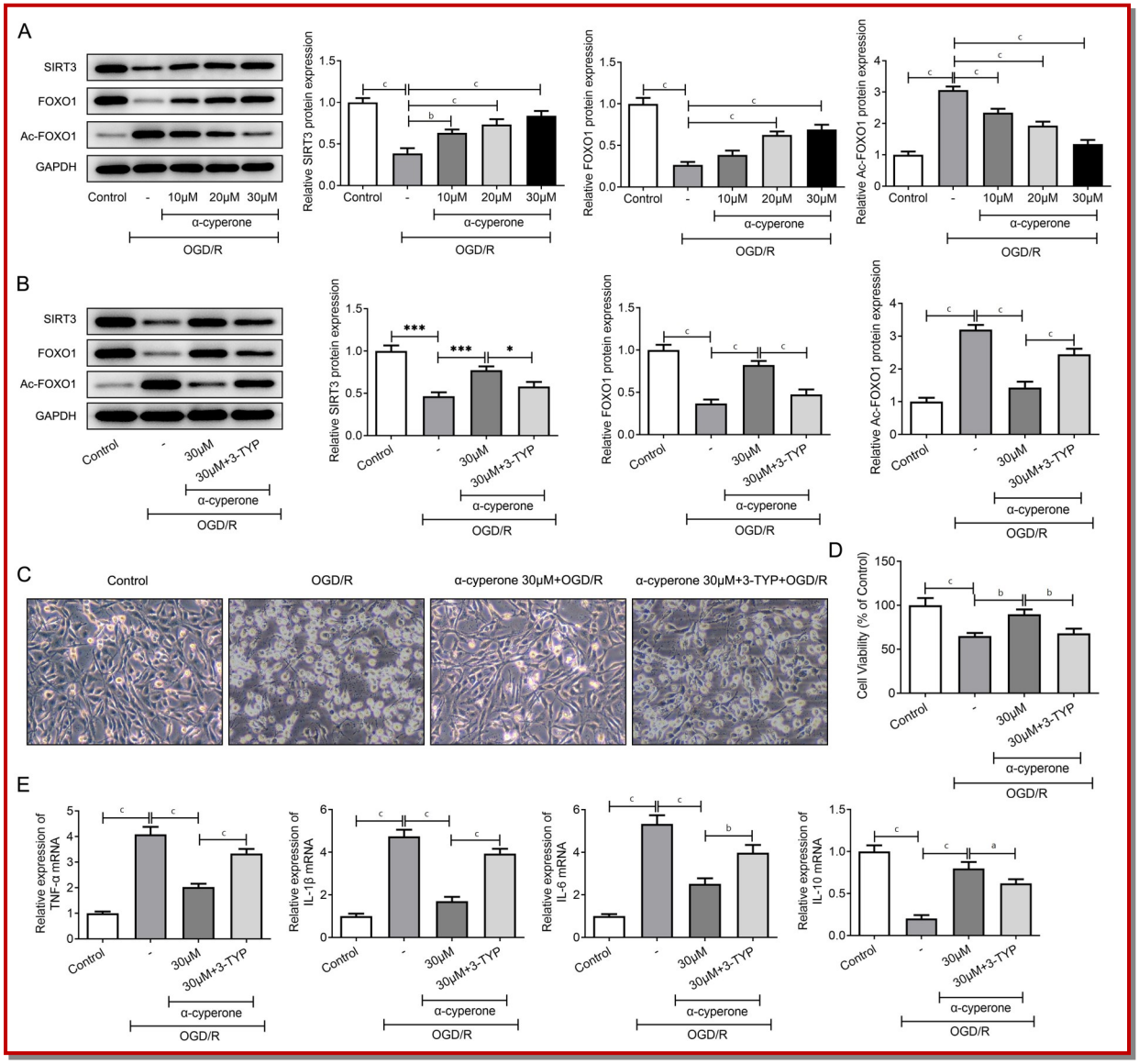

Figure 3: α-Cyperon activates SIRT3/FOXO1 signaling and 3-TYP breaks the effect of α-cyperon on neuronal viability and inflammation. The expression levels of SIRT3, acetyl-FOXO1 and FOXO1 were determined using western blotting (A). Following the cells were co-treated with α-cyperone and 3-TYP, the expression levels of SIRT3, acetyl-FOXO1, and FOXO1 were determined using western blotting (B). Cell viability and morphology were evaluated (C-D). The levels of inflammatory factors in the cells were determined using RT-qPCR analysis (E). [a]p<0.05, [b]p<0.01, [c]p<0.001

concentration-dependent manner. It can be concluded from the above that α-cyperone can maintain OGD/R-induced neuronal viability, inhibit inflammation, and oxidative stress depending on different doses, however, the signal pathway through which it may go is not yet known. A previous study points out that the presence of SIRT3 can reduce cerebral I/R injury (Zhao et al., 2018), and another study considers that SIRT3 can promote FOXO1 deacetylation and stabilize FOXO1 expression (Zhang et al., 2013). In addition, FOXO1 has been studied to be acetylated by SIRT1, an enzyme of the same family as SIRT3, and participate in cerebral I/R injury (Wang et al., 2020). Therefore, we hypothesized that α-cyperone might activate SIRT3, promote FOXO1 deacetylation, and then regulate cerebral I/R injury. 3-TYP was employed to treat cells to explore the effect of 3-TYP on the pharmacological effects of α-cyperon. The experimental results indicated that after the cells were treated with 3-TYP, the protective effect of α-cyperone on the cells was blocked. This suggested that α-cyperone promoted the SIRT3/FOXO1 signaling, and alleviated the damage to cells induced by OGD/R. Nevertheless, this article is limited to *in vitro* experiments, and the function of α-cyperone on cerebral I/R requires further *in vivo* studies.

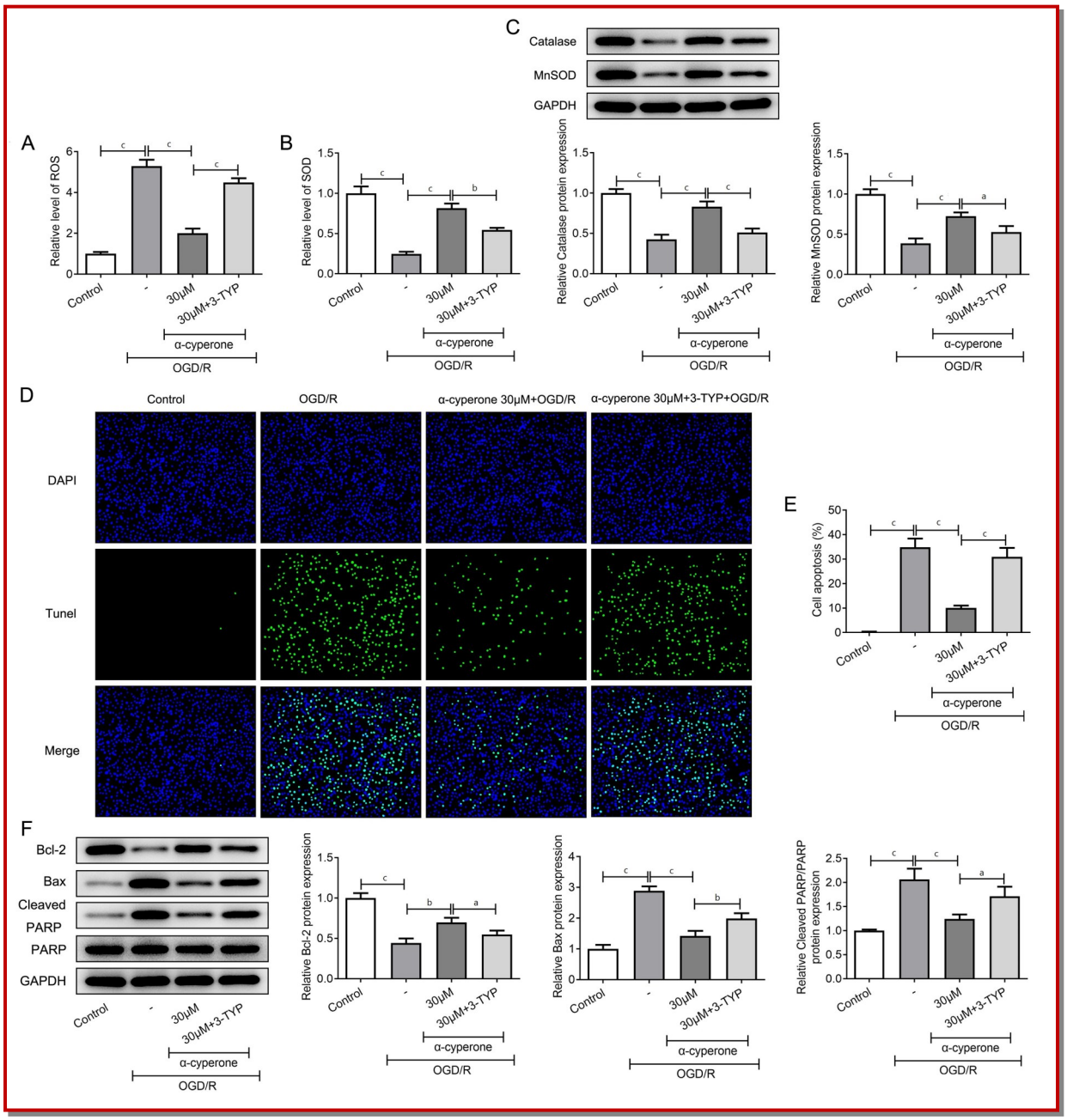

Figure 4: 3-TYP breaks the effect of α-cyperon on neuronal oxidative stress and apoptosis. The level of ROS (A) and SOD in the cells was determined using assay kits (B). The levels of catalase and MnSOD were determined using Western blotting (C). The effect of α-cyperone on the level of cell apoptosis was evaluated using TUNEL assay (D-E) and Western blotting (F). Magnification, x200. [a]p<0.05, [b]p<0.01, [c]p<0.001

Conclusion

α-Cyperone activated SIRT3 and FOXO1 deacetylation, and alleviated OGD/R-induced cell inflammation, oxidative stress, and apoptosis. These findings explore the pharmacological effects of α-cyperone and provide a theoretical basis for its application in the treatment of cerebral I/R.

Financial Support

Self-funded

Conflict of Interest

Authors declare no conflict of interest

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