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Compound 21 Attenuates Isoflurane-Induced Injury in Neonatal Rat Hippocampal Neurons and Primary Rat Neuronal Cells by Upregulating METTL3

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Background: Isoflurane, as an anesthetic drug, has a neurotoxic effect on the developing brain tissue. Compound 21 (C21) has been reported to be neuroprotective and ameliorate stroke effects. However, the mechanism by which C21 protects against nerve damage remains unclear.

Methods: Animal and cellular models of brain injury were constructed using isoflurane (ISO) in neonatal SD rats and primary rat neuronal cells (PRNCs). After treatment with C21, the ultrastructure and morphology of the hippocampus in the model rats were assessed using the transmission electron microscope and H&E staining. Methylation or apoptosis-related genes or proteins were examined using immunohistochemistry, RT-qPCR, and Western blot. The levels of inflammatory factors were monitored using the ELISA kits. m6A modification was analyzed by Dot blot and MeRIP-qPCR. Cell proliferation and apoptosis were also tested using Edu and TUNEL staining.

Results: C21 suppresses apoptosis and inflammation and improves hippocampal morphology in ISO-induced neonatal rats. Mechanistically, C21 upregulates m6A modification, PPAR-a, BCL-2, and METTL3 in ISO-induced neonatal rats and ISO-treated PRNCs. C21 promotes cell proliferation, enhances BCL-2 m6A modification, and reduces inflammation by upregulating METTL3 by upregulating METTL3 in ISO-treated PRNCs.

Conclusion: These findings suggest that C21 enhances neuronal cell survival and morphology and up-regulates methylation and Bc1-2 levels, potentially offering a therapeutic strategy for neuroprotection in clinical settings, particularly in cases of neurotoxic exposure. The mechanism may be related to the upregulation of METTL3.

Keywords: compound 21, isoflurane, METTL3, anesthesia, neuron

Introduction

Anesthetic-induced developmental neurotoxicity (AIDN) refers to the degenerative changes in the developmental brain caused by general anesthetics, which affect brain development, learning, and cognitive function.[1] Isoflurane (ISO), as a commonly used inhaled anesthetic, has been shown to affect specific neural substrates, particularly in the hippocampus, where it disrupts key neural circuits related to memory and cognition.[2] Studies indicate that ISO exposure in the developing brain can lead to dysfunction in hippocampal neurons, impacting areas such as the CA1 and CA3 regions and the dentate gyrus.[3,4] This damage to hippocampal circuits is associated with cognitive impairments and learning deficits in animal models.[5] Moreover, ISO exposure is linked to the activation of the tryptophan-kynurenine metabolic

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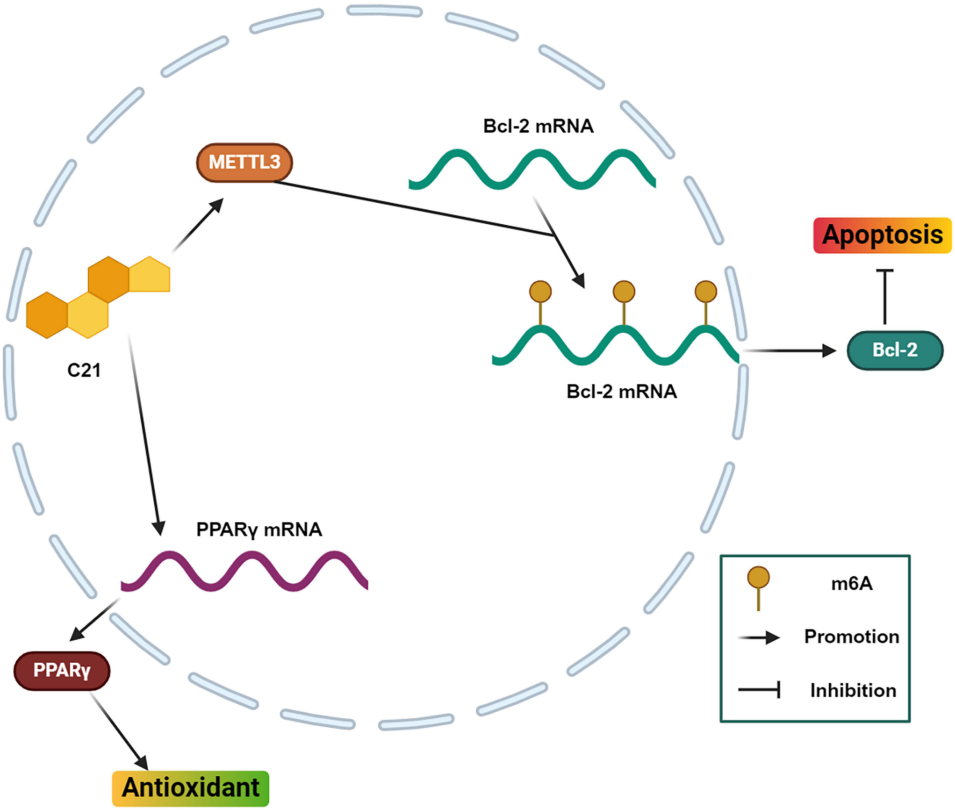
Graphical Abstract



system, which plays a significant role in modulating stress and fear-related pathways.[6] Studies indicated that ISO can induce neuronal apoptosis, oxidative stress, and mitochondrial dysfunction, which collectively contribute to neurodegeneration and cognitive dysfunction.[7–10] Given the widespread use of ISO in pediatric anesthesia, there is a critical need for therapeutic strategies that can mitigate its adverse effects on the developing brain.

Several neuroprotective compounds have been investigated to counteract ISO-induced neurotoxicity.[11,12] For example, melatonin can attenuate oxidative stress and mitochondrial dysfunction and have potential for neuroprotective potential.[13,14] However, its bioavailability and inconsistent findings pose challenges. Another compound, dexmedetomidine, has demonstrated anti-inflammatory and anti-apoptotic effects in ISO-induced models, though its sedative effects and narrow therapeutic window limit its clinical application.[15] Similarly, erythropoietin and lithium have shown varying degrees of efficacy in preclinical studies but are limited by side effects or lack of specificity.[16,17] These limitations underscore the need for novel, more effective therapeutic agents that offer better neuroprotection with fewer side effects.

Compound 21 (C21), an AT2R agonist, is a newly developed non-peptide, an orally active, specific, and highly selective compound that exerts vasodilatory, anti-inflammatory, anti-fibrotic, and antiproliferative effects.[5,18–20] Recent studies have highlighted the neuroprotective potential of AT2R activate C21 may preserve hippocampal structure and function in models of neurological injury.[21,22] By specifically targeting AT2R, C21 reduces inflammation and promotes cell survival without the sedative side effects of other neuroprotective agents such as dexmedetomidine.[23,24] Its oral bioavailability also makes it more convenient for clinical use. However, while these features make C21 an attractive therapeutic candidate, the exact mechanisms underlying its neuroprotective effects remain to be fully elucidated.

N6-methyladenine (m6A), as an internal modification, can participate in gene regulation by multiple mRNA processes in eukaryocytes.[25–27] This methylation modification is achieved by m6A methyltransferase complexes such

as METTL3.[28] m6A modification plays a key role in multiple biological processes (metabolism, oxidative stress, proliferation).[29–31] Studies also suggested that m6A-associated regulatory proteins play crucial roles in the cerebral cortex, synaptic function, axonal regeneration, neural stem cell self-renewal, and cerebellar development.[32–34] Based on this, the present study hypothesizes that the neuroprotective effects of C21 may be related to the regulation of gene methylation through m6A modification.

In this study, ISO was applied to induce neuronal damage in primary rat neuronal cells (PRNCs) and neonatal rats, assessing the potential of C21 to ameliorate the resulting cellular and structural changes. Related experiments were conducted to explore the mechanism by which C21 ameliorates ISO-induced cell morphology changes. This research aims to provide a new theoretical and experimental basis for developing therapeutic strategies to alleviate ISO-induced neuronal damage, offering potential insights into improving clinical outcomes in pediatric patients exposed to anesthetics.

Materials and Method

Experimental Animals

Forty Sprague-Dawley (SD) rats (7 days of age, body weight 22–30 g, half male and half female) were purchased from Shanghai Super B&K Laboratory Animal Co., Ltd. (Shanghai, China; license number 2013–0016). All rats were housed in an environment consisting of 24±2°C, 50–60% humidity, 12h light/12h dark cycle, and artificially fed chow and water. The chosen timeframe for conducting these experiments is based on prior studies indicating that 7-day-old rats display developmental brain characteristics sensitive to anesthetic-induced neurotoxicity, particularly in the hippocampal region.[35,36] This experiment was approved by the Animal Ethics Committee of Guizhou Medical University (Guiyang, China). The experiments were conducted following the Guide for the Care and Use of Laboratory Animals as the National Institutes of Health recommended.

Animal Grouping

The rats were inhaled 1.5% ISO (Sigma-Aldrich) for 3 h for 3 consecutive days to establish the brain injury model. Rats were injected intraperitoneally with 30, 60, and 90 μg/kg of C21 (Sigma-Aldrich; Merck KGaA, cat. no. C160) 30 min before ISO inhalation. The sham group was treated similarly to the other experimental groups but without exposure to ISO or C21. Experimental rats were randomly divided into five groups: sham group, model group (ISO treatment), low-dose C21 treatment group (the model rats were injected with 30 μg/kg C21 intraperitoneally), medium-dose C21 treatment group (the model rats were injected with 60 μg/kg C21 intraperitoneally), high-dose C21 treatment group (the model rats were injected with 90 μg/kg C21 intraperitoneally). There were 8 rats in each group. Sample size determination was based on a power analysis ($\alpha = 0.05$, power = 0.80), which confirmed that a minimum of 8 animals per group was sufficient to detect a statistically significant effect size in the primary outcomes of this study. The young rats were sacrificed by amputation, and fresh left and right hippocampus tissues and serum were collected for brain damage detection in young rats.

Transmission Electron Microscopy (TEM)

The right fresh hippocampal tissue (about 1mm) was fixed in 2.5% glutaraldehyde for 2 h and fixed in 1% osmic acid for 1.5 h. After washing with PBS, the tissues were dehydrated with gradient ethanol and acetone. The tissues were soaked in a mixture of epoxypropane and epoxy resin Epon812 (1: 1), embedded at 37 ° C. The slides were stained with 2% toluidine blue and placed on a 200-mesh copper mesh for uranyl acetate-lead citrate staining. The ultrastructural changes were observed under TEM (80 kV; JEM-1230; JEOL, Ltd., Tokyo, Japan).

Neurobehavioral Evaluation

The neurological impairment was assessed using the modified neurological severity score (mNSS),[37] a scoring system that evaluates motor, sensory, reflex, and balance functions to quantify impairment severity. Prior to evaluation, animals received treatment according to a standardized protocol. Following treatment, animals underwent a series of standardized tests to assess neurological function and assign an mNSS score. The evaluation included tests for motor function (assessing ability to walk on a narrow beam and limb placing), sensory responses (response to touch and whisker stimulation), reflex testing (pinna and corneal reflexes), and balance function. Scores range from 0 to 18, with higher

scores indicating greater impairment: scores from 1 to 6 indicate mild impairment, marked by minor deficits; scores from 7 to 12 represent moderate impairment, with noticeable effects on movement and coordination; and scores from 13 to 18 denote severe impairment, with profound deficits in motor and sensory functions that significantly impact daily activities.

Immunohistochemistry (IHC)

The slides were deparaffinized using xylene and hydrated using gradient ethanol. The slides were subjected to high-pressure antigen repair with citrate and treated with 3% H_2O_2 after washing. After blocking with goat serum (Gibco) for 1 h, the slides were incubated with anti-BCL-2 (1: 20; Abcam) or anti-Caspase-3 (2 μg/mL; Abcam) at 4 °C overnight. The slides were added with biotin-labeled secondary antibody (1: 400; Abcam) for 1 h after washing. After staining with 8 mg/mL 3-amino-9-ethylcarbazole (AEC), the slides were stained with hematoxylin (Sigma-Aldrich) for 2 min, and differentiated using hydrochloric acid ethanol. The expressions of BCL-2 and Caspase-3 were observed using a light microscope (Olympus) after dehydration, transparency, and sealing.

Cell Culture

Primary rat neuronal cells (PRNCs) consisting of 40% neurons and 60% astrocytes were purchased from BrainBit (E18 rat cortex; Springfield, IL, USA). PRNCs were grown in neural medium (NbActive 4, BrainBits) including 2% B27 and 2 mm L-glutamine at 37°C with 5% CO_2.

Cell Treatment

The neuronal injury model was induced by 1.2 mm ISO for 6 h. Then, the model cells were treated with 0.1, 1, and 10 μM C21 for 48 h. METTL3 shRNAs (sh-METTL3) were obtained from Genepharma (Shanghai, China). METTL3 overexpression plasmid (OE-METTL3) was purchased from Biosyntech (Suzhou, China). PRNCs were treated with ISO and C21 and then transfected with sh-METTL3 or OE-METTL3 using Lipofectamine^{TM} 3000 (Invitrogen) for 48 h according to the kit instruction.

Dot Blot

DNA was extracted from the treated PRNCs using the Cellular Genomic DNA extraction kit based on the instructions. m6A was determined in line with the instructions provided by the EpiQuik m6A RNA Quantification Assay Kit (Abcam).

RT-qPCR

The left fresh hippocampal tissues were ground, and total RNA was harvested using TRIzol (Invitrogen, MA, USA). After purification, total RNA was reversed using the reverse transcription kit (Takara, Tokyo, Japan). qPCR was conducted using SYBR Green qPCR Master Mix (DBI Bioscience). The levels of PPAR-α, METTL3, and BCL-2 were quantified through the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Total protein was isolated from the processed PRNCs and the ground fresh hippocampal tissues using RIPA buffer (Pierce, IL, USA). The proteins were quantified, separated by electrophoresis, and transferred to the PVDF membrane (Millipore, USA). After blocking with 5% skim milk powder for 1 h, the proteins were soaked in anti-PPAR-α (1: 1000; Abcam, Cambridge, UK), anti-METTL3 (1: 1000; Abcam), anti-BCL-2 (1: 1000; Abcam), and anti-GAPDH (1: 1000; Abcam) overnight at 4 °C. After processing with secondary antibody (1: 2000; Abcam), the proteins were visualized by ECL (GE Healthcare, Piscataway, NJ).

Elisa

Based on the manufacturer's instruction, the levels of IL-6, IL-2, TNF-α, IL-4, and IL-10 in rat serum were analyzed using the corresponding ELISA kits (Jiangmei Biotechnology, Jiangsu, China).

Edu Staining

The treated PRNCs (4×10^3 cells/well) were inoculated in 96-well plates and incubated in 50 μM medium containing Edu (1000: 1) for 1 h. The cells were fixed with 4% paraformaldehyde for 30 min, and processed with 2 mg/mL glycine for 5 min. The cells were added with 0.5% Triton X-100 in PBS (100 μL) for 10 min after washing. Then the cells were processed with 1 mg/mL DAPI (Sigma-Aldrich). The stained cells were detected by fluorescence microscopy (Olympus IX71; Olympus Corporation).

MeRIP-qPCR

RNA Extraction Kit extracted total RNA from the left fresh hippocampal tissues, and the extracted total RNA was purified using the PolyA mRNA Purification Kit. Antibodies against BCL-2 and IgG were added to the immunoprecipitation (IP) buffer and conjugated to protein A/G magnetic beads by incubation for 1 h. The complexes were added to an IP buffer containing ribonuclease inhibitor and protease inhibitor, and incubated at 4°C overnight. The eluted RNA was extracted and purified by the phenol-chloroform method. The level of BCL-2 m6A was analyzed using RT-qPCR.

Statistical Analysis

The measurement data was denoted as mean ±SD from 3 independent experiments. The data was analyzed using SPSS 21.0 (IBM, SPSS, Chicago, IL, USA) with one-way ANOVA. Assumptions of normality and homogeneity of variance were assessed through Shapiro–Wilk and Levene's tests, respectively, prior to conducting the ANOVA. When assumptions were not met, data transformations or non-parametric tests were considered to ensure the accuracy and validity of the results. $P < 0.05$ signified the statistical difference.

Results

C21 Reduces the mNSS Score of ISO-Induced Neonatal Rats

As shown in Figure 1, the rats in the sham group had no postoperative neurological deficits (the mNSS score is 0); relative to the sham group, the mNSS score was markedly increased in the model group; C21 treatment reduced dramatically the mNSS score of the model rats. C21 treatment, particularly at the 60 μg/kg concentration, significantly reduced the mNSS score in ISO-induced rats, suggesting a dose-dependent neuroprotective effect of C21.

C21 Improves Neuronal Cell Morphology and Inhibits Apoptosis in ISO-Induced Neonatal Rats

To verify the role of C21 on ISO-mediated neurotoxicity in neonatal rats, neonatal SD rats were exposed to 1.5% ISO and intraperitoneally injected with 30, 60, and 90 μg/kg C21. TEM results exhibited that the ultrastructure of hippocampal cells in the sham group was normal with clearly defined cell membranes, endoplasmic reticulum, and well-preserved mitochondrial cristae; in the ISO-induced model group, significant ultrastructural damage was apparent, the hippocampal cells of rats were shrunken and reduced in size, the cell membranes and endoplasmic reticulum membranes were dissolved, the cytoplasmic coagulation and density increased, the rough endoplasmic reticulum was blurred, the mitochondrial cristae were reduced and blurred, and the nuclear membranes showed wavy wrinkles; C21 treatment improved the ultrastructure of the rat hippocampus, with improved membrane integrity and clearer endoplasmic reticulum and mitochondrial structures, especially the medium concentration group (Figure 2A). Besides, IHC data uncovered that ISO exposure led to decreased BCL-2 and increased Caspase-3 expression in the hippocampus, indicating enhanced apoptosis; C21 treatment, especially 60 μg/kg C21 markedly elevated BCL-2 level and reduced Caspase-3 level in the neurons of model rats (Figure 2B-E).

C21 Upregulates the Levels of m6A Modification, PPAR-A, BCL-2, and METTL3 and Reduces the Inflammatory Response in ISO-Induced Neonatal Rats

Subsequently, the possible mechanism by which C21 ameliorated neuronal cell damage was explored in ISO-induced neonatal rats. The data of Dot blot revealed that the m6A modification level in the neurons of the model group (ISO-induced neonatal

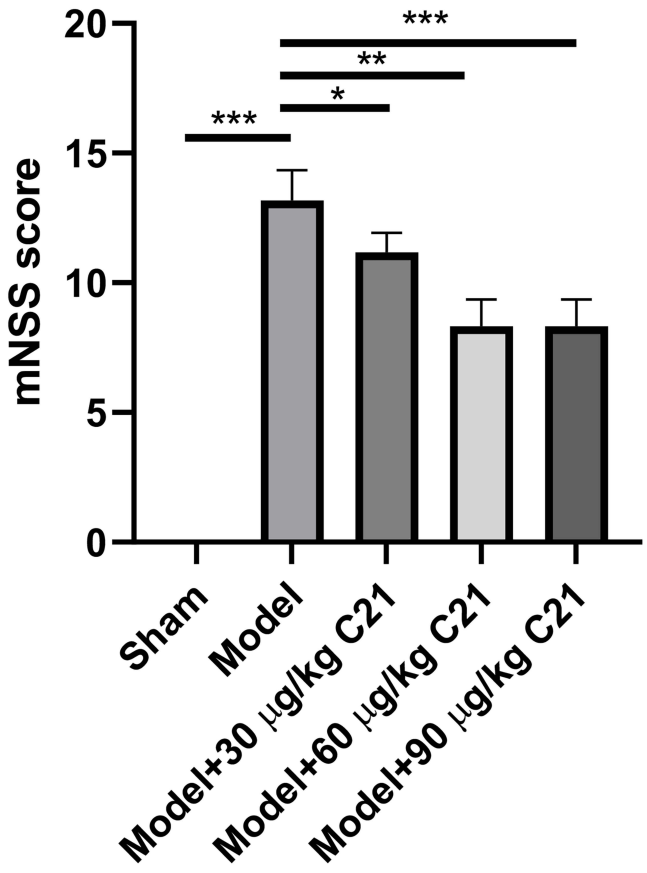


Figure 1 Effect of C21 on neurofunctional behavior of ISO-induced neonatal rats. A brain injury model was established in neonatal SD rats with 1.5% ISO. mNSS score of the model rats was assessed after treatment with C21. *P<0.05, **P<0.01, ***P<0.001.

rats) was lower than that of the sham group, and C21 treatment, particularly at 60 μg/kg, increased the m6A modification level in the neurons of ISO-induced neonatal rats (Figure 3A). RT-qPCR data denoted that the mRNA levels of PPAR-α, METTL3, and BCL-2 in hippocampal neurons of the model group were lower than those in the sham group; C21 reversed the decrease of PPAR-α, METTL3, and BCL-2 expressions mediated by ISO in rat hippocampal neurons (Figure 3B). Western blotting data also disclosed that C21 treatment up-regulated PPAR-α, METTL3, and BCL-2 proteins in the hippocampal neurons of ISO-induced neonatal rats (Figure 3C and D). Additionally, the data displayed that ISO increased IL-6, IL-2, and TNF-α levels and decreased IL-4 and IL-10 levels in hippocampal neurons of neonatal rats, which also could be notably attenuated by C21, especially 60 and 90 μg/kg C21 (Figure 3E).

C21 Induces the Proliferation of ISO-Treated PRNCs

In vitro experiments were further performed to verify the effect of C21 on the proliferation of ISO-treated PRNCs. Edu staining results denoted that Edu-positive cells in the model group (ISO treatment) were lower than those in the control group; C21, especially at medium and high concentrations, dramatically increased the number of Edu-positive cells in ISO-treated PRNCs, suggesting a dose-dependent effect on cell proliferation (Figure 4).

C21 Upregulates m6A Modification, PPAR-A, BCL-2, and METTL3 in ISO-Treated PRNCs

To further explore the possible mechanism by which C21 attenuates ISO-induced neuronal cell injury, PRNCs were obtained and treated with ISO and C21. Western blotting data signified that the downregulation of PPAR-α, METTL3, and BCL-2 expressions mediated by ISO could be prominently reversed by C21, especially 1 μM and 10 μM C21 (Figure 5A and B). Moreover, Dot blot data exhibited that ISO observably reduced the relative 6mA abundance in

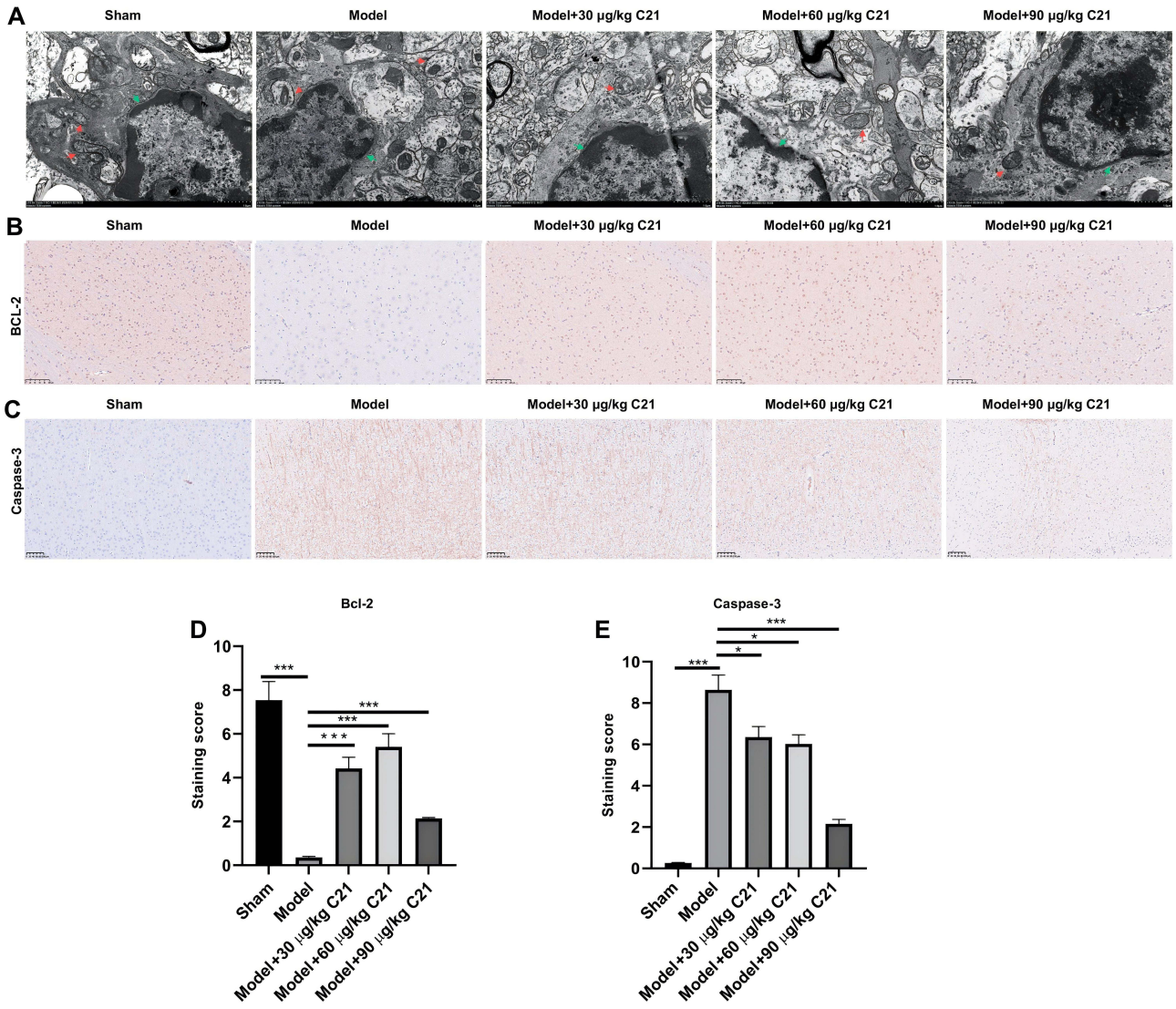


Figure 2 C21 improves neuronal cell morphology and inhibits apoptosis in ISO-induced neonatal rats. Neonatal SD rats were exposed to 1.5% ISO to establish a model of brain injury. The model rats were intraperitoneally injected with 30, 60, and 90 μg/kg C21. (**A**) TEM observed the ultrastructure of the hippocampus. IHC assay was applied to determine the changes in BCL-2 (**B**) and Caspase-3 (**C**) expressions in rat brain tissues, magnification, 200×. The quantitative analysis of BCL-2 (**D**) and Caspase-3 (**E**) is based on the results of IHC. **P*<0.05, ****P*<0.001. Green arrow, endoplasmic reticulum. Red arrow, mitochondria.

PRNCs; C21, especially 1 μM C21, markedly increased the 6mA abundance in ISO-treated PRNCs (Figure 5C). Based on the above results, 60 μg/kg C21 was selected as the appropriate concentration for PRNCs treatment.

C21 Accelerates Proliferation by Upregulating METTL3 in ISO-Treated PRNCs

Further, the present study further investigated whether METTL3 could be involved in C21-mediated neuronal cell proliferation in ISO-treated PRNCs. After treatment with ISO and C21, PRNCs were transfected with sh-METTL3 or OE-METTL3. Edu staining results manifested that C21 promoted the proliferation (Edu-positive cells) of ISO-treated PRNCs; cell proliferation mediated by C21 was reversed by METTL3 silencing and further enhanced by METTL3 overexpression, suggesting that METTL3 upregulation plays a key role in C21-induced proliferation (Figure 6).

C21 Attenuates Inflammation and Increases BCL-2 m6A by Upregulating METTL3 in ISO-Treated PRNCs

Meanwhile, ELISA data indicated that the reduction of IL-6, IL-2, and TNF-α levels and the increase of IL-4 and IL-10 levels mediated by C21 also could be attenuated by METTL3 silencing and enhanced by METTL3 overexpression in

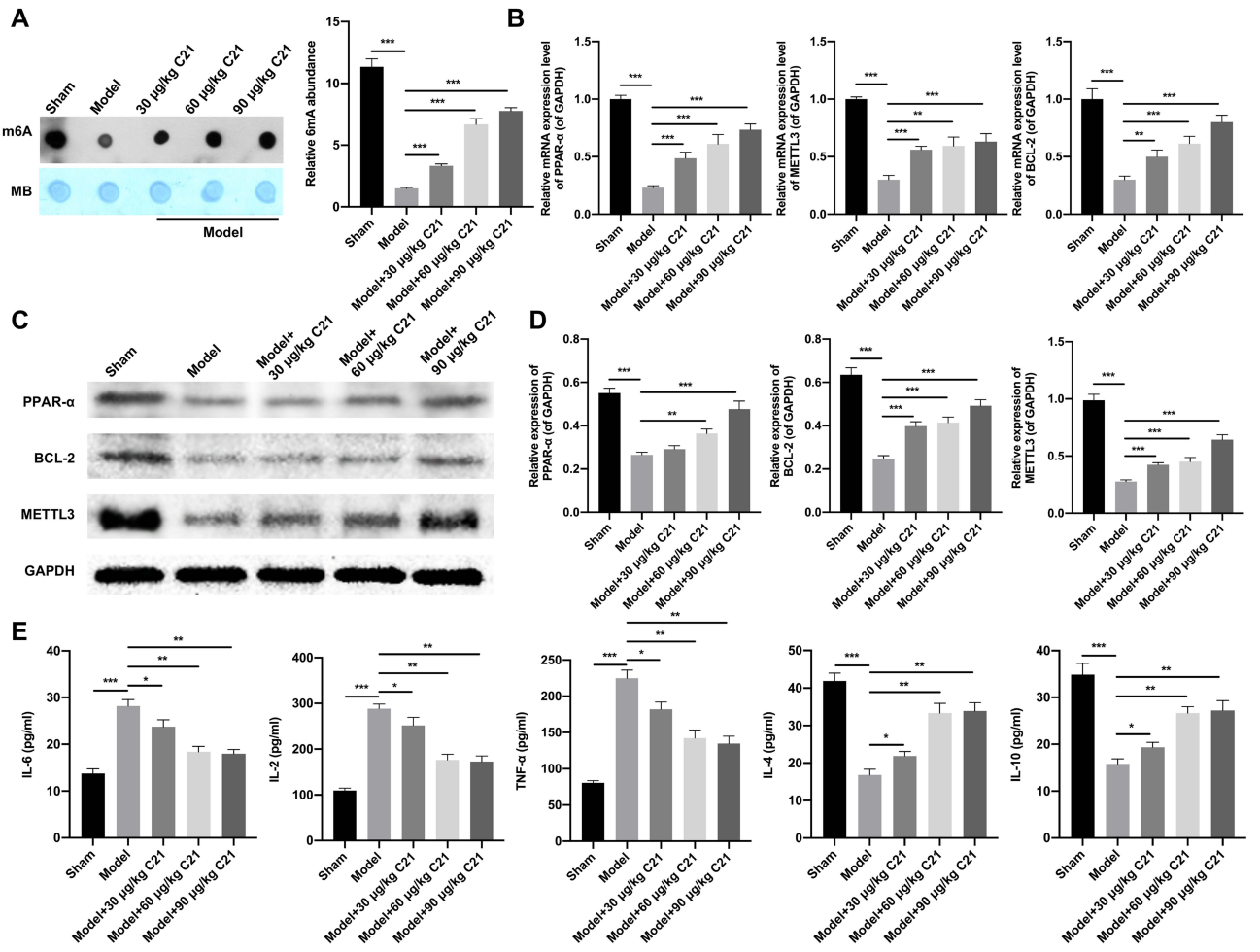


Figure 3 C21 upregulates the levels of m6A modification, PPAR-A, BCL-2, and METTL3 and reduces the inflammatory response in ISO-induced neonatal rats. The ISO-induced neonatal rats were processed with 30, 60, and 90 μg/kg C21 by intraperitoneal injection. (**A**) The m6A modification level was assessed using a Dot blot. (**B**) The expressions of PPAR-α, METTL3, and BCL-2 were monitored through RT-qPCR. (**C**) Western blotting analysis of PPAR-α, METTL3, and BCL-2 protein expressions in the hippocampus. (**D**) Quantitative analysis of Western blot results. (**E**) ELISA kits were adopted to analyze the levels of inflammation-related factors (IL-6, IL-2, TNF-α, IL-4, and IL-10). **P*<0.05, ***P*<0.01, ****P*<0.001.

ISO-treated PRNCs (Figure 7A). Besides, MeRIP-qPCR data uncovered that C21 treatment increased the m6A modification level of BCL-2 in ISO-treated PRNCs, METTL3 silencing reduced and METTL3 overexpression raised the m6A modification level of BCL-2, which C21 induced in ISO-treated PRNCs (Figure 7B).

Discussion

The study's findings demonstrated that ISO induced apoptosis, altered hippocampal neuron morphology in neonatal rats, reduced proliferation, and increased inflammation in PRNCs. These results aligned with previous studies showing that ISO exposure could compromise hippocampal integrity and potentially contribute to cognitive decline in developing brains.[9,38,39] Our study expanded upon this understanding by investigating the potential of C21 as a therapeutic agent for mitigating ISO-induced neuronal damage.

ISO, as a non-barbiturate anesthetic, has the characteristics of rapid onset, controllability, and few side effects, and is widely applied in clinical surgery.[40] Several studies verified that ISO is highly neurotoxic to the rat hippocampus.[9,38,39] This study aimed to further search for possible therapeutic agents for ISO-induced hippocampal neuronal damage. SD rats are outbred, highly sensitive to neurodevelopment and widely applied in neuroscience and neurotoxicology.[41] In this study, SD rats on the 7th day after birth and PRNCs were selected for the study. The results of the current study demonstrate that ISO could induce apoptosis and change the morphology of hippocampal neurons in neonatal rats, and

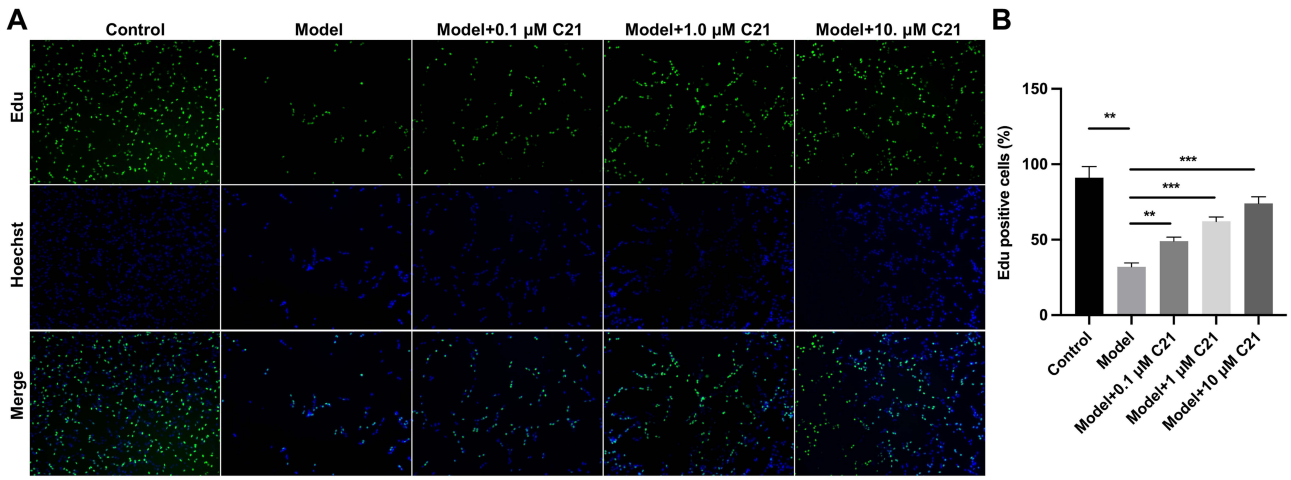


Figure 4 C21 induces proliferation of ISO-treated PRNCs. After inducement with ISO for 6 h, PRNCs were treated with 0.1, 1, and 10 μM C21. (**A**) The cell proliferation ability was assessed using Edu staining, magnification, 200×. (**B**) Quantitative analysis of Edu-positive cells. ***P*<0.01, ****P*<0.001.

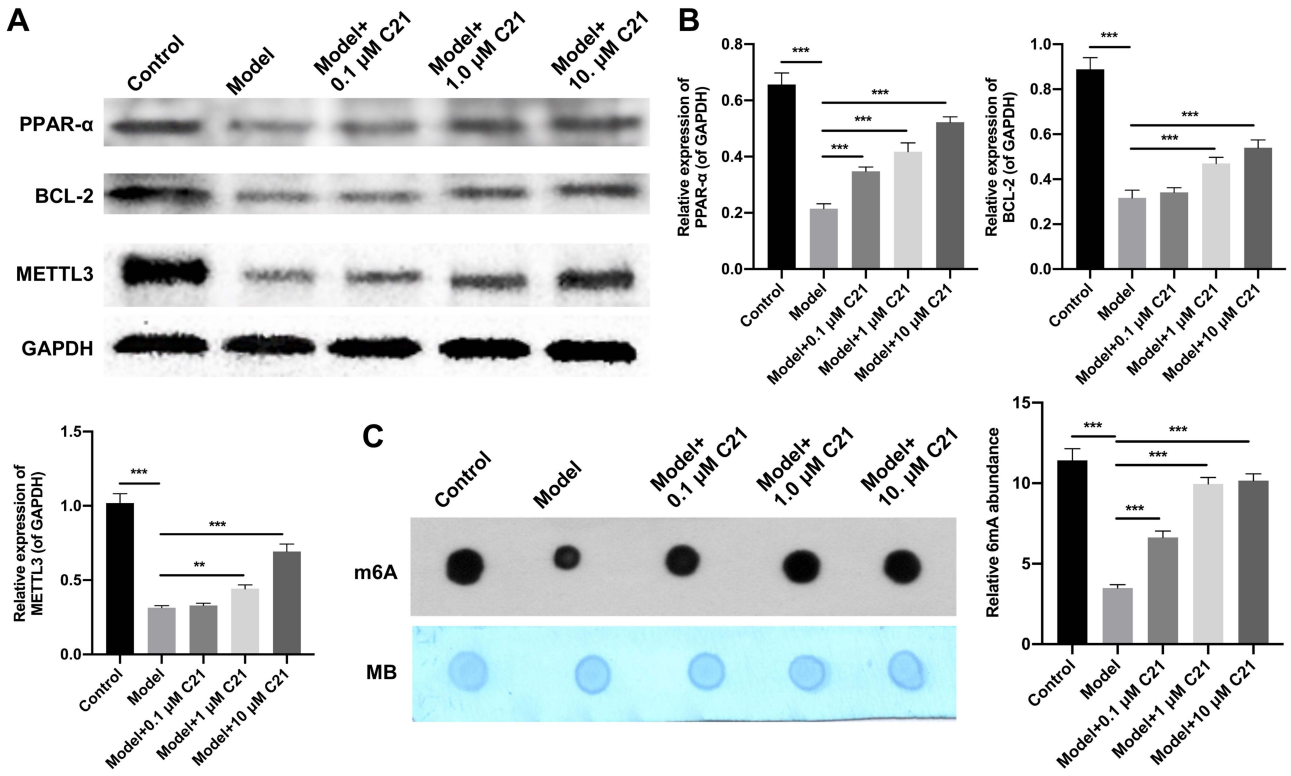


Figure 5 C21 upregulates m6A modification, PPAR-A, BCL-2, and METTL3 in ISO-treated PRNCs. After inducement with ISO for 6 h, PRNCs were treated with 0.1, 1, and 10 μM C21. (**A**) Western blotting analysis for the examination of PPAR-α, METTL3, and BCL-2 expressions in the treated PRNCs. (**B**) The PPAR-α, METTL3, and BCL-2 to GAPDH ratios were quantitatively analyzed based on the Western blot results. (**C**) Dot blot revealed the change in m6A modification level, and the relative 6mA abundance was quantified. ***P*<0.01, ****P*<0.001.

ISO could also weaken proliferation and enhance inflammation of PRNCs. These data further confirm that ISO can cause hippocampal neuronal damage.

C21, as a selective ligand for AT2R, can reduce oxidative stress, improve cerebral blood flow, and promote vasodilation. Studies indicated that C21 has a significant ameliorative effect on neuronal damage.[42–44] For example, C21 can improve ischemic neuronal damage by up-regulating VEGF;[42] After ischemia/reperfusion injury, C21 can exert a neuroprotective effect through interleukin (IL)-10 to enhance neuronal survival and reduce apoptosis;[43] C21 can

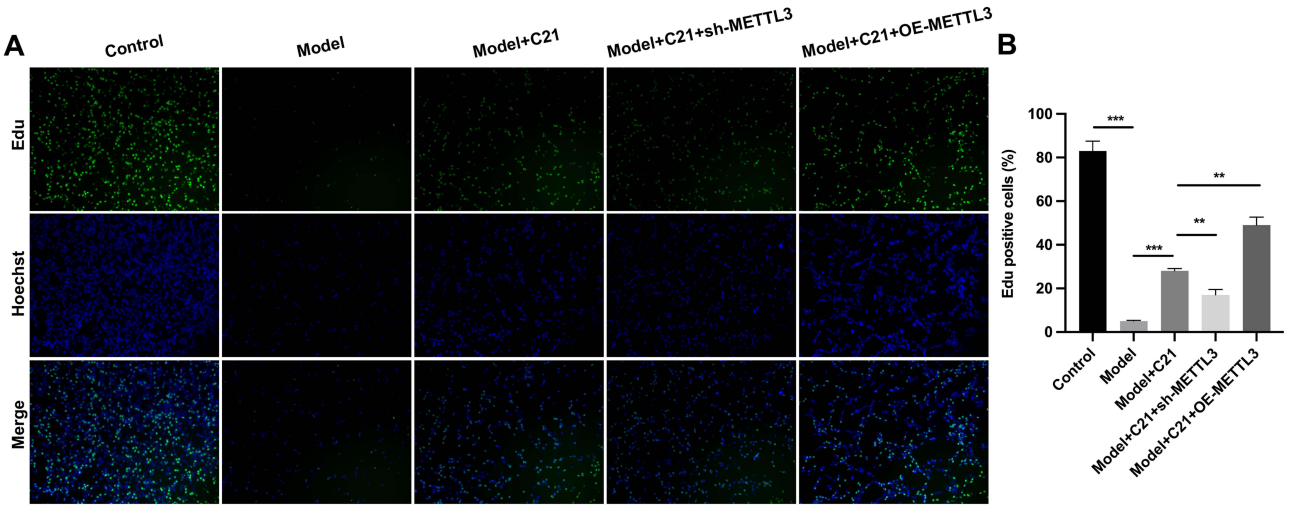


Figure 6 C21 accelerates proliferation by upregulating METTL3 in ISO-treated PRNCs. After treatment with ISO and C21, PRNCs were transfected with sh-METTL3 or OE-METTL3. (**A**) Edu staining was carried out to identify cell proliferation in the treated PRNCs, magnification, 200×. (**B**) Quantitative analysis of Edu-positive cells. ***P*<0.01, ****P*<0.001.

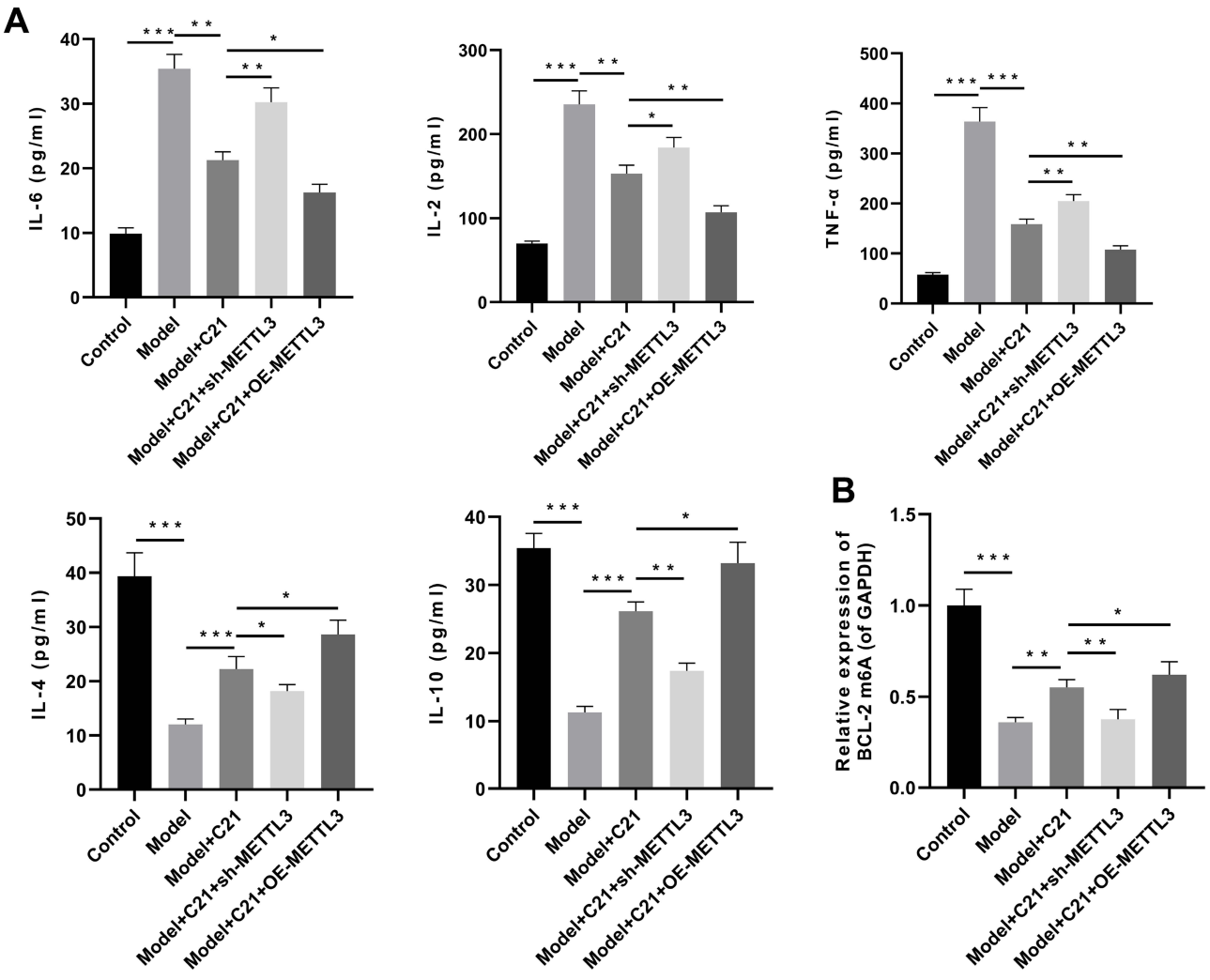


Figure 7 C21 attenuates inflammation and increases BCL-2 m6A by upregulating METTL3 in ISO-treated PRNCs. After treatment with ISO and C21, PRNCs were transfected with sh-METTL3 or OE-METTL3. (**A**) The expression levels of IL-6, IL-2, TNF-α, IL-4, and IL-10 were credited using the ELISA kits. (**B**) The m6A modification level of BCL-2 was monitored using MeRIP-qPCR. **P*<0.05, ***P*<0.01, ****P*<0.001.

improve neuronal survival after stroke in mice.[44] Therefore, it is vital to elucidate the mechanism by which C21 alleviates the toxic effects of ISO on developing brain tissue. Our previous study further revealed that C21 attenuated ISO anesthesia-induced apoptosis in neonatal rat hippocampal neurons and improved cell morphology.[45] The current data further indicate that C21 significantly inhibits hippocampal neuronal apoptosis and improves neuronal cell morphology in ISO-induced neonatal rats. Besides, C21 can accelerate the proliferation of ISO-treated PRNCs.

Our previous study also demonstrated that C21 could enhance the expressions of PPAR-α and BCL-2 in ISO-induced neonatal rat hippocampal neurons.[45] PPAR-α is a nuclear receptor involved in regulating fatty acid metabolism and inflammatory response.[46] BCL-2 is an anti-apoptotic protein that plays a role in maintaining cell survival and preventing apoptotic processes.[47] The current data further reveal that C21 could upregulate PPAR-A and BCL-2 in ISO-induced neonatal rats and PRNCs. Thus, this study suggests that C21 attenuates ISO-induced damage to hippocampal neurons.

Moreover, this study identified METTL3 as a key mediator in the neuroprotective effects of C21. METTL3 is one of the most critical methyltransferases for m6A methylation.[48] Studies proved that loss or overexpression of METTL3 alters the total methylation level of m6A and plays a key role in nerve injury.[49,50] In our study, the data first confirmed that C21 could increase m6A modification and METTL3 levels in the hippocampal neurons of ISO-induced neonatal rats and ISO-treated PRNCs. Meanwhile, we proved that C21 could accelerate proliferation and inhibit inflammation by upregulating METTL3 in ISO-treated PRNCs. Moreover, C21 could increase BCL-2 m6A through METTL3 in ISO-treated PRNCs. This represents a novel insight into the potential mechanisms by which C21 mitigates ISO-induced neurotoxicity.

This study has certain limitations. First, the relatively small sample size (40 rats) may affect the generalizability of the findings. Future studies with larger sample sizes are essential to validate the observed effects and provide a more robust understanding of C21's therapeutic potential. Second, while this study focused on short-term outcomes following C21 treatment, long-term studies assessing cognitive and behavioral impacts are needed to assess the clinical relevance of C21, particularly for pediatric anesthesia applications. Investigating potential lasting effects or side effects would be valuable in confirming C21's safety and efficacy. Third, although METTL3 was identified as a key factor in C21's neuroprotective mechanism, further studies should explore other signaling pathways and downstream effects involved in neuroprotection. Elucidating these pathways, especially those involved in inflammation and apoptosis would enhance the understanding of C21's full therapeutic potential. Additionally, while comparisons were made between treated and untreated groups, future studies could include additional control groups with varied ISO doses and alternative neuroprotective agents to better establish C21's relative effectiveness. Such studies would provide a clearer picture of C21's neuroprotective capacity in comparison to other treatments.

Another limitation of this study is the use of primary rat neuronal cells derived from E-18 rats for in vitro experiments, while neonatal rats (day 7) were used for in vivo studies. This difference in developmental stages may introduce variability when comparing cellular responses to C21 between in vitro and in vivo models. However, using E-18 cells provides the advantage of high viability and consistency in culture, which facilitates detailed molecular analysis under controlled conditions. This approach allows for a deeper understanding of the cellular mechanisms of C21, which can then be validated in the more complex biological environment of the neonatal rat model. Future studies should consider using age-matched cell models to ensure greater consistency and improve the translational relevance of the findings.

Conclusions

In this study, we propose for the first time that C21 enhances the survival of ISO-induced hippocampal neuronal cells by upregulating METTL3, thereby alleviating ISO-induced neuronal damage. Additionally, the study highlighted the role of m6A methylation in the neuroprotective effects of C21, providing a novel perspective on the epigenetic regulation of nerve injury. Moreover, the varying doses of C21 used in this study suggest potential dose-dependent effects, with medium concentrations showing notable efficacy. Further investigation into the dose-response relationship, particularly at lower doses, could help identify optimal therapeutic concentrations for clinical applications, ensuring safety and efficacy. Thus, these findings provide a theoretical basis for C21 as a potential therapeutic agent for treating ISO-induced nerve injury.

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Disclosure

Yaping Shen and Yijiu Wu are regarded as co-first authors. The authors report no conflict of interest.

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