Overexpression of miR-221-3p effecting cell proliferation, apoptosis and inflammation by targeting Toll-like receptors 4 in propofol-induced rat astrocytes

Type
Research paper

Keywords
TLR4, cell cycle, apoptosis, MyD88

Abstract
Introduction
Growing evidence indicated that propofol has neurotoxic effects on the brains of developing rodents, leading to neuronal cell death, neurodegeneration, and brain injury.

Material and methods
Ectopic miR-221-3p was transfected into rat astrocytes, and Cell Counting Kit-8 assay and flow cytometry were performed to evaluate cell growth and apoptosis. The mRNA levels of Toll-like receptors 4 (TLR4), nuclear factor kappa B, interleukin-6, interleukin-1β, myeloid differentiation primary response 88 (MyD88), caspase-3, caspase-12, STAT3, and GRP78 were detected using quantitative real-time polymerase chain reaction. The proteins of TLR4 and MyD88 were determined using Western blotting. The association between miR-221-3p and TLR4 was measured using Dual-Luciferase Reporter Assay (Promega Corporation, Wisconsin, USA). Then, siTLR4 was transfected with 293T cells to study the role of TLR4 in astrocytes with propofol treatment.

Results
The miR-221-3p expression in rat astrocytes was markedly suppressed by propofol treatment. The miR-221-3p mimics transfection in propofol-treated astrocytes effectively reduced the suppressive effect of propofol on astrocyte growth, repressed the propofol-induced apoptosis in rat astrocytes, and decreased the cell number during the G2–M phase. The expression of MyD88 and TLR4 was induced by propofol, whereas the transfection of miR-221-3p mimics dramatically reduced these genes expression at the mRNA and protein expression. After that, TLR4 was found to be target of miR-221-3p using Dual-Luciferase Reporter Assay. Furthermore, knockdown of TLR4 could suppress the apoptosis rate in propofol-treated astrocytes.

Conclusions
This study revealed that miR-221-3p might prevent astrocytes from propofol-induced damage by targeting TLR4.
Overexpression of miR-221-3p effecting cell proliferation, apoptosis and inflammation by targeting 
*Toll-like receptors 4* in propofol-induced rat astrocytes

**Running Title:** miR-221-3p affected astrocytes proliferation

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**Abstract**

**Background:** Growing evidence indicated that propofol has neurotoxic effects on the brains of developing rodents, leading to neuronal cell death, neurodegeneration, and brain injury. Besides, microarray data indicated that the miR-221-3p in mRNA and protein expression levels was negatively altered in propofol-treated rat
astrocytes; however, the effect of miR-221-3p on propofol-treated astrocytes remains unclear. Thus, we aimed to evaluate the function and mechanism of miR-221-3p on propofol-treated astrocytes.

**Method:** Ectopic miR-221-3p was transfected into rat astrocytes, and Cell Counting Kit-8 assay and flow cytometry were performed to evaluate cell growth and apoptosis. The mRNA levels of Toll-like receptors 4 (TLR4), nuclear factor kappa B, interleukin-6, interleukin-1β, myeloid differentiation primary response 88 (MyD88), caspase-3, caspase-12, STAT3, and GRP78 were detected using quantitative real-time polymerase chain reaction. The proteins of TLR4 and MyD88 were determined using Western blotting. The association between miR-221-3p and TLR4 was measured using Dual-Luciferase Reporter Assay (Promega Corporation, Wisconsin, USA). Then, siTLR4 was transfected with 293T cells to study the role of TLR4 in astrocytes with propofol treatment.

**Results:** The miR-221-3p expression in rat astrocytes was markedly suppressed by propofol treatment. The miR-221-3p mimics transfection in propofol-treated astrocytes effectively reduced the suppressive effect of propofol on astrocyte growth, repressed the propofol-induced apoptosis in rat astrocytes, and decreased the cell number during the G2–M phase. The expression of MyD88 and TLR4 was induced by propofol, whereas the transfection of miR-221-3p mimics dramatically reduced these genes expression at the mRNA and protein expression. After that, TLR4 was found to be target of miR-221-3p using Dual-Luciferase Reporter Assay. Furthermore, knockdown of TLR4 could suppress the apoptosis rate in propofol-treated astrocytes.

**Conclusions:** This study revealed that miR-221-3p might prevent astrocytes from propofol-induced damage by targeting TLR4.

**Keywords:** apoptosis, cell cycle, TLR4, MyD88
Background

Propofol (2, 6-diisopropyl phenol) is used as general intravenous anesthetic worldwide, due to its fast onset and low postoperative adverse reactions (1, 2). Previous studies showed that propofol also was regarded as anesthetic for pediatric individuals and pregnant woman (3, 4). However, in great majority clinical children practices, propofol is considered as an off-label choice (5). More and more studies have found that propofol might cause neurotoxicity and neurogenic damage. At present, there is a lack of evidence to support the safe use of propofol (6, 7). Besides, astrocytes are the most abundant glial cells in the central nervous system (8), which provide critical support for brain development, neuronal differentiation, and neurotransmitter homeostasis in developing and adult CNS (9, 10). Thus, the potential role of propofol in developing anesthesia strategies may be reconsidered.

MicroRNAs (miRNAs) are short non-coding RNAs (18–23 nt) that bind to the seed sequences within the 3’UTR region of target mRNAs to regulate translational repression or degradation of target mRNAs (11). In addition, miRNAs dysregulation was associated with neurodegenerative disease (10, 12). Some miRNAs are related to inflammatory pathways, such as miR-155, miR-146a, miR-21 and miR-124 (13). Besides, studies have proven that miRNAs affect astrocyte cell cycle and their conversion into neurons, such as miR-146a (14), miR-338 (15), and miR-143-3p (16). In addition, previous studies indicated that miR-221-3p is related to inflammatory conditions (17, 18). Up to now, the miR-221-3p expression mainly occurs in pathological conditions associated with cancer, such as thyroid cancer (19), non-small cell lung cancer (20) and breast cancer (21). Moreover, Feng et al. (22) indicated that miR-221-3p could be used as a biomarker for mediated the activation in astrocytes. Toll-like receptors (TLRs) are a family of transmembrane pattern recognition receptors that are mainly expressed on immune cells (23). TLRs could induce a series of cell signal transduction pathways, release inflammatory mediators, and activate the adaptive immune system (24). In addition, previous studies
have indicated that miR-221-3p could mediate the inflammatory response in human umbilical vein endothelial cells through the target gene TLR4 (17).

However, few studies have focused on the function of miR-221-3p on propofol-treated astrocytes through TLR4. Thus, in our study, we explore the function of miR-221-3p in astrocytes treated by propofol. First, the Cell Counting Kit-8 assay and flow cytometry were carried out to assess the astrocytes cell growth and apoptosis after transfecting miR-221-3p. After that, siTLR4 was transfected with 293T cells to study the role of TLR4 in astrocytes with propofol treatment.

Results

Propofol downregulated mRNA expression of miR-221-3p expression in rat astrocytes

We isolated primary astrocytes from rat brain and ensured cell reliability using immunostaining for GFAP, a typical marker of astrocytes (25, 26). Most cells in the microscope field displayed GFAP positivity, demonstrating that the rat astrocytes were successfully obtained (Figure 1A). Besids, qRT–PCR results revealed that propofol treatment significantly reduced the miR-221-3p expression ($p < 0.05$) (Figure 1B).

MiR-221-3p attenuates propofol-induced decrease in cell viability in rat astrocytes

To probe the action of miR-221-3p on astrocytes during propofol treatment, miR-221-3p was ectopically imported into propofol-treated astrocytes. After transfecting astrocytes with miR-221-3p mimic and NC mimic for 48 h, qRT–PCR results showed that miR-221-3p expression dramatically improved in the miR-221-3p mimic-transfected group as contrasted with the NC mimic and controls ($p < 0.01$), demonstrating that miR-221-3p was successfully delivered into astrocytes (Figure 2A). As shown in Figure 2B, CCK-8 assay results indicated that the cell viability in the propofol group decreased contrasted with that in the blank group, while the cell viability in the the propofol + miRNA mimic group was dramatically upregulated compared with
that of the propofol group or the propofol + miRNA NC group especially 72 h and 96 h after propofol addition 

\( p < 0.05, \ p < 0.01 \).

**MiR-221-3p regulates apoptosis and cell cycle in propofol-treated cells**

As indicated in **Figure 3A**, the apoptosis rates in the propofol + miR-221-3p mimic were decreased when compared with propofol and propofol + miRNA NC group \( (p < 0.05) \), indicating that miR-221-3p overexpression restored propofol-induced apoptosis in rat astrocytes. In addition, a significant increase or decrease in the proportion of cells in the G2/M phase was observed in cells transfected with miR-221-3p mimics compared with their respective NCs (**Figure 3B**). Overall, these data indicated that miR-211-3p downregulation could block the cell cycle in the G2/M phase.

**MiR-221-3p affected propofol-induced astrocyte apoptosis**

To study the mechanism of miR-221-3p on cell proliferation and apoptosis, we examined the related genes including Proliferating cell nuclear antigen (PCNA), GFAP, caspase-12, nuclear factor kappa B (NF-κB), interleukin-6 (IL-6), and interleukin-1β (IL-1β), MyD88 (Myeloid Differentiation Factor 88), Toll-like receptor 4 (TLR4), Signal transducer and activator of transcription 3 (STAT3), caspase-3, and glucose-regulated protein 78 (GRP78). As shown in **Figure 4A**, the results indicated that PCNA mRNA level in the propofol + miR-221-3p mimic group was significantly improved when compared with propofol group \( (p < 0.05) \), while that has no significance compared with propofol+ miRNA NC group. In addition, the mRNA expression of GFAP, **STAT3** and **GRP78** in propofol + miR-221-3p mimic group has no significance when compared with propofol group and propofol+ miRNA NC group. The mRNA expression of caspase-12 was significantly decreased when contrast with propofol group and propofol+ miRNA NC group \( (p < 0.05) \). Moreover, the mRNA expression of **NF-κB**, **MyD88**, **TLR4**, **IL-6** and **IL-1β** in in propofol + miR-221-3p mimic group was markedly reduced when contrasted with other three groups \( (p < 0.01) \). Besides, we further examined the protein expression of TRL4,
MyD88 and NF-κB, the results indicated that the overexpression of miR-221-3p dramatically decreased cellular protein levels of TLR4, MyD88 and NF-κB (p50) (Figure 4B). This result indicated that the overexpression of miR-221-3p could reverse the expression of TLR4, MyD88 and NF-κB (p50) in astrocytes induced by propofol.

**TLR4 was a target of miR-221-3p**

We found that the miR-221-3p expression trend was opposite to that of TLR4 (Figure 1B and Figure 4A). Complementary sequences were observed between TLR4 3’UTR and miR-221-3p when combined with bioinformatics prediction (Figure 5A). The association between miR-221-3p and TLR4 was detected using the Dual-Luciferase Reporter Assay System. Ectopic expression of miR-221-3p significantly decreased the TLR4 3’UTR-WT luciferase activity (Figure 5B).

**Knockdown of TLR4 could suppress the apoptosis rate in propofol-treated astrocytes**

To explore the function of TLR4 in propofol-treated astrocytes, TLR4 was knocked down by transfecting 293T cells that were transfected with siTLR4. The results of qRT–PCR indicated that TLR4 expression was markedly decreased after the knockdown of TLR4-1, TLR4-2, and TLR4-3 (Figure 6A). We selected TLR4-1 as the final siRNA sequence for the following experiments. Besides, TLR4 knockdown cells markedly suppressed apoptosis compared with the cells in the siRNA controls (p < 0.01) (Figure 6B and C). In addition, we further examined the protein related apoptosis, including bax, bcl-2, caspase 3 and caspase 9 using western blot. The results indicated that the expression of bax and caspase 3 in propofol + siTLR4 group were decreased when compared with propofol + siNC group, while the expression of bcl-2 and caspase 9 were improved when compared with propofol + siNC group.

**Discussion**
In this study, we found that the miR-221-3p expression in rat astrocytes was markedly suppressed by propofol treatment. Besides, the overexpression of miR-221-3p could improve the propofol-induced astrocytes viability and decrease its apoptosis and the cell number during the G2–M phase. In addition, the transfection of miR-221-3p mimics could dramatically reduce the mRNA and protein expression of MyD88, TLR4 and NF-κB in propofol-induced astrocytes. After that, TLR4 was found to be target of miR-221-3p using Dual-Luciferase Reporter Assay. Furthermore, knockdown of TLR4 could suppress the apoptosis rate in propofol-treated astrocytes. Previous studies have reported that miRNA could regulate the nerve damage caused by propofol (27). Besides, Feng et al (22) has indicated that miR-221-3p could be regarded as a biomarker to regulated the activation of astrocyte. However, the specific effect and potential mechanism of miR-221-3p on propofol-treated astrocytes have not yet been explored. In this study, our results indicated that miR-221-3p was an important regulator of rat astrocyte proliferation in response to propofol treatment. First, miR-221-3p expression was decreased in the treatment of propofol in rat astrocytes. Furthermore, we indicated that miR-221-3p overexpression can improve cell growth and suppress astrocyte apoptosis, which were consistent with the miRNA profile data (28, 29). Moreover, to probe the pathway associated with miR-221-3p, we conducted qRT–PCR detection to quantify apoptosis-related gene expression. The results indicated that the overexpression of miR-221-3p in propofol-induced astrocytes could significantly reduce the mRNA expression of NF-κB, MyD88, TLR4, IL-6, and IL-1β. For further bioinformatics analysis, we found TLR4 was the target of miR-221-3p, which was identified using Dual-Luciferase Reporter Assay. TLR4 is a membrane receptor that can recognize a variety of stimuli and activate related signal transduction pathways when stimulated, thereby promoting the expression of inflammatory factors (30, 31). After knockdown of TLR4, we observed that TLR4 knockdown cells markedly suppressed the propofol-induced apoptosis rate, implying that miR-221-3p affects astrocyte proliferation that
was not related to ER-stress signals. Zhu et al (17) indicated that miR-221-3p regulated HUVECs apoptosis, inflammation through the target of TLR4. Quero et al (32) indicated that miR-221-3p was considered as a regulator of inflammatory M2 macrophage function induced by TLR4. The above reports suggested that miR-221-3p could mediate apoptosis and inflammation via the target of TLR4. Therefore, our studies inferred that miR-221-3p could improve the damage of CNS that induced by propofol through regulate the expression of TLR4.

There are some limitations in our research. First, due to the characteristics of the multiple targets that propofol exists on the cell, we are currently unable to determine the way propofol enters the cell to perform its function. Therefore, our research cannot clarify the exact mechanism by which is-miRNA regulates the neurotoxicity caused by propofol. The TLR4 mediated NF-KB pathway need to be validated in propofol induced astrocytes, which could provide a better understanding for the function of miRNA in propofol induced astrocytes. In addition, in order to clarify whether propofol is toxic to the developing brain, it is necessary to conduct *in vivo* propofol exposure experiments in newborn animals.

**Conclusions**

In summary, we found the overexpression of miR-221-3p could improve the viability of propofol-induced astrocytes and decrease its apoptosis rate. In addition, the results indicated that miR-221-3p may protect astrocytes from propofol-induced damage by targeting *TLR4*.

**Methods**

**Animals**
Pregnant Sprague–Dawley (SD) rats were obtained from Shanghai Laboratory Animal Center (Shanghai, China).

The rats were raised using adequate food and water under constant temperature (23°C ± 1°C) and maintained on an adequate light–dark cycle for 12 h.

**Cell cultures**

Twenty-four hours after birth, newborns were euthanized. The mice were disinfected with 75% alcohol. We removed the cerebral cortex from the skull and carefully dissected the meninges. Moreover, we cut the brain tissues into small pieces, which were digested using 4-mL Hanks' Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, MA, USA). Then, 1-mL DNase, 4-m mL HBSS, and 1-mL trypsin (2.5%) were added to 10 mL volume and the specimens underwent a water bath at 37°C for 15 min.

Isolated cells were transferred into a fresh 50-m mL tube and were washed using modified Eagle’s medium (MEM) (Whitaker Bioproduct, MD, USA) with 10% characterized horse serum (Hyclone, UT, USA) to stop trypsin activity. Dissociated cells were collected through centrifugation at 1000 rpm for 5 min. Then, the MEM containing 10% horse serum was added and re-suspended. 293T cells were obtained from the Cell Bank of Shanghai Academy of Health Sciences (Shanghai, China), cultured in Gibco Dulbecco’s Modified Eagle Medium (DMEM) (ME100202P1; Thermo Fisher Scientific, MA, USA), and added to 10% fetal bovine serum (FBS) (10091-148; Thermo Fisher Scientific, MA, USA), which were incubated in a 5% CO₂ at 37°C.

**Identification of astrocytes**

The glial fibrillary acidic protein (GFAP) expression in astrocytes was identified using immunofluorescence staining. In short, we cultured the cells at a density of 4 × 10⁵ cells/mL in poly-L-lysine-coated coverslips. After attaching the cells to the flask, immunofluorescence staining was applied for identifying astrocytes. The fresh 4% paraformaldehyde (Solarbio, Beijing, China) was prepared for fixing the cells at 4°C for 30 min. After that, we washed the cells for once time using phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin.
(Corning Inc., Corning, NY, USA) and the cells were lysed using 0.1% Triton X-100 (Solarbio, Beijing, China) at room temperature for 20 min. Then, we washed the cells with PBS for three times, and blocked using 3% BSA in PBS for 1 h, and combined with the primary antibody against GFAP (rabbit anti-GFAP, 1:800; ab7260, Abcam, Cambridge, UK). The cells were incubated overnight in a humid room at 4°C. The cells were washed with PBS for three times, and then which were incubated with donkey anti-rabbit IgG-488 (Sigma-Aldrich, MO, USA) at 37°C for 1.5 h in the dark and stained with 4,6-diamidino-2-phenylindole (Cell Signaling Technology, Danfoss, Massachusetts, USA). All images were obtained through a fluorescence microscope (Optika, Ponteranica, Bergamo, Italy) with appropriate filters.

**Cell processing**

Astrocytes were treated with 10-μM propofol (the propofol-treated group), and astrocytes not treated with isopropanol were used as controls (the control groups). After 48 h, we discarded the culture medium, then added 1-mL TRIzol (Thermo Fisher Scientific, MA, USA) to each group of cells, and stored at −80°C.

**Cell transfection**

A mimic negative control miRNA (NC mimics) and miR-221-3p mimic were obtained from GenePharma (Shanghai, China). The NC mimics and miR-221-3p mimic were transfected into astrocytes by Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, MA, USA) based on commercial guidelines. Briefly, then, 20 nM of miR-221-3p-mimics or miR-221-3p-NC was mixed with the transfection agent in Opti-MEM (Thermo Fisher Scientific, MA, USA) for 10 min, after that added to the astrocytes seeded on 6-well plates. The cells were replaced with fresh DMEM 5 h after transfection and further cultured for 48 h until the experiments.

**Quantitative real-time polymerase chain reaction (qRT–PCR)**

The propofol-treated and control cells were collected for total RNA extraction using commercial RNAiso Plus (Takara, Dalian, China) following the user’s manual. The first-strand cDNA was reverse transcribed based on
Reverse transcription of miRNAs was performed using PrimeScript™ RT Master Mix (Takara, Dalian, China) at 65°C for 5 min. PCR reactions were conducted using SYBR® Green Realtime PCR Master Mix (TOYOBIO, Osaka, Japan). PCR protocols were pre-denatured at 95°C for 3 min, denatured for 30 cycles at 95 °C for 30 s, annealed at 60°C for 30 s, extension at 72°C for 30 s, and finally extended at 72°C for 8 min. Primers of the indicated genes were synthesized using Genscript (Nanjing, China), and the primer sequences are presented in Table 1. The intracellular levels of U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were referred as the internal controls for miRNAs and mRNAs, respectively. The relative levels of genes and miRNAs were calculated as $2^{-\Delta\Delta CT}$ (33).  

| Primer | Sequence(5’-3’) |
|--------|----------------|
| PCNA-rF | GCTTCGGGCAGCACCATACTACTAAAAT |
| PCNA-rR | CGCCTTCAGGAATTTGCGTGTCAT |
| GFAP-rF | GGTGTTGAGTGCCCTTCGTAATTAG |
| GFAP-rR | GGGACACTTTTCAGCTCCATTCT |
| TLR4-rF | GATGGCTCAGACATGGCAGTTTC |
| TLR4-rR | CACTCGAGTAGGTGTTTCTGCTAA |
| NF-κB-rF | GCACGGATGACAGAGGCATGTATAAGG |
| NF-κB-rR | GGGGATGATCTCCTTCATCTGTCTG |
| IL-6-rF | AAGAAAGACAAAGGCCAGAGTC |
| IL-6-rR | CACAAAATGATATGCTTAGGC |
| IL-1β-rF | AGGCTGACAGACCACAAAGAGAT |
| IL-1β-rR | CTCCACGGGCAAGCATAGGTAA |
| MyD88-rF | TGGTGGTCTTTCTGACAT |
| MyD88-rR | GATCAGTGCTCTGTTGGA |
| caspase-3-rF | GGATTTGAGACAGACAGTGG |
| caspase-3-rR | CACGGGATCTGTTTCTTGC |
| caspase-12-rF | GGAAGGTTAGGCAAGAGT |
| caspase-12-rR | GTAGAAGTAGCGTGTCATA |
| MyD88-rF | GCTGACTTGGGAGCCTGATTCT |
| MyD88-rR | ATGGGCTGGGTTGGAGTAAA |
| STAT3-rF | CACCTGGGATTGAGAGTAGAC |
Cell viability assay

Cell viability was detected through Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China). Thus, the cells (100 μL/well) were seeded in 96-well plates for 24 h, 48 h, 72 h, and 96 h, respectively. Then, we added 10-μL CCK-8 solution to the cells and incubated in an incubator at 37°C for 2 h. The absorbance at 450 nm (OD450) was evaluated using a microplate reader (Bio-Rad, Hercules, USA). Four groups were constructed: the propofol group, propofol + miRNA mimic group, propofol + miRNA NC group, and blank control group.

Flow cytometry

After propofol treatment for 48 h, we collected the cells for apoptosis and cell cycle assay. The cells for apoptosis assay were first re-suspended in 1 × Binding Buffer and incubated with 5-μl APC-Annexin V and 5-μl propidium iodide (PI) for 20 min in the dark at 25°C. After gently mixing the cells with 400-μl 1×Binding Buffer, the apoptotic rate was quantified using FACSCalibur (BD Biosciences, NJ, USA) within 1 h. In the cell cycle assay, the harvested cells were re-suspended and fixed by 5-ml pre-cooling 70% ethanol overnight at 4°C. After that, we washed the cells with precooled PBS and re-suspended the cells in 0.3-ml PBS using a gentle pipette. RNase A was added to the cells at a final concentration of 50 μg/mL and digested at 37°C for 30 min. The cell cycle of the cell samples was measured using flow cytometry after the final incubation with another 5-μl PI at 4°C for 15 min.

| Primer | Sequence |
|--------|----------|
| STAT3-rR | AGGAATCGGCTATATTGCTGGT |
| GRP78-rF | TCAGCCCAACCTGAACAT |
| GRP78-rR | CAAACTTCTCGGCGTCAT |
| rno-miR-221-3p-RT | GTCGTATCCAGTGCGAGGGTTCGAGTCTCGACTGAGCATCGACGAAACC |
| rno-miR-221-3p-F | GCCGCACCTCACTTGACGTGTC |
| rno-U6-RT | CGCTTCAGAATTGCGGTCAT |
| rno-U6-F | GCTTCGCGACCATATAACTAAAAT |
| rno-U6-R | CGCTTCAGAATTGCGGTCAT |
| GAPDH-rF | AGACACGGCGCATTCTTTCGT |
| GAPDH-rR | CTTGCGGTGGTGAGTCAT |
Western blotting

All collected cells were washed twice with precooled PBS, lysed in a radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China), then treated with ultrasound, and centrifuged at 10,000 g at 4 °C for 20 min. The protein amount was quantified using a bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, MA, USA). The total protein was isolated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to a polyvinylidene difluoride film (Millipore, Watford, UK). The membrane was immunoblotted overnight at 4°C with anti-Toll-like receptor 4 (TLR4) (1:300; Abcam, MA, USA), anti-myeloid differentiation primary response 88 (MyD88) (1:500; Abcam, MA, USA), NF-κB (p50) (66535-1-IG, Proteintech), BAX (50599-2-IG, Proteintech), Bcl-2 (26593-1-AP, Proteintech), CASPASE3 (19677-1-AP, Proteintech), CASPASE9 (10380-1-AP, Proteintech) and anti-GAPDH (Santa Cruz Biotechnology, CA, USA). After washing in 1 × tbst for three times, the horseradish peroxidase-labeled secondary antibody (Jackson ImmunoResearch, PA, USA) was used for detection at room temperature for 1 h. The protein bands were measured using an enhanced chemiluminescent system (Merck Millipore, Watford, UK). Jackson ImmunoResearch.

Dual-Luciferase Reporter Assay

We used miRwalk online software (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2) to predict the target gene of miR-221-3p that can regulate TLR4. Besides, we synthesized TLR4 3’UTR fragment containing a miR-221-3p-binding site and mutant binding site and cloned it into pGL3 promoter plasmid (Omega, USA, D6950-02) to prepare TLR4 3’UTR-WT. Lipofectamine 2000 (11668-027; Invitrogen, Thermo Fisher Scientific, MA, USA) was used to co-transfect 293T cells with miR-221-3p or miR-NC mimics. The relative light units were detected using the Dual-Luciferase Reporter Assay System (Promega).
Transient transfection with small interfering RNA (siRNA)

The method of transfecting siRNA was according to a previous study (34). 293T cells were transfected with siRNA–TLR4 to knock down the TLR4 gene, and the empty plasmid siNC was the control. Stable transfected 293T cells were collected using the Zeocin screening method. The mRNA expression of TLR4 was determined using qRT–PCR.

Statistical analysis

Statistical Package for the Social Sciences (version 21.0; IBM Corp., Armonk, NY, USA) was used to statistically process the experimental data. The quantified results are shown as mean ± standard deviation (SD). One-way analysis of variance was used to analyze the difference between any two groups. \( P < 0.05 \) indicated statistical significance, and \( P < 0.01 \) indicated extremely significant differences.

List of abbreviations

CCK-8: Cell Counting Kit-8
CNS: central nervous system
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GFAP: glial fibrillary acidic protein
miRNAs: MicroRNAs
MyD88: myeloid differentiation primary response 88
NC mimics: negative control miRNA
PBS: phosphate-buffered saline
PI: propidium iodide
RIPA: radioimmunoprecipitation assay
SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel

TLR4: Toll-like receptors 4

TLRs: Toll-like receptors

Declarations

Ethics approval and consent to participate

Approval was obtained from the ethics committee of Jilin University. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Shan-Shan Yu and Shuang Qi were responsible for the conception and design of the research, and drafting the manuscript.

Zinan Li and Shuang Qi performed the data analysis and interpretation.

Shuang Qi performed the statistical analysis.
All authors have read and approved the manuscript.

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Not applicable.

Conflict of interest

There was no conflict of interest in the study.

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Figure captions

Figure 1. Propofol Treatment reduces miR-221-3p Expression in Purified Rat Astrocytes

A. Purified cells were adopted for immunostaining of glial fibrillary acidic protein to verify the extraction efficiency of astrocytes. B. Isolated rat astrocytes were treated with 10-μM propofol for 48 h, miR-221-3p quantification was examined using qRT–PCR. *P < 0.05 contrasted to the control group.

Figure 2. Overexpression of miR-221-3p attenuates Cell Proliferation Induced by Propofol Treatment

A. Synthetic miR-221-3p mimic and miR-221-3p NC were transfected into purified rat astrocytes, and delivery efficiency was examined through qRT–PCR. B. Purified astrocytes divided into four groups were harvested at indicated time points for cell viability detection using CCK8. ** P < 0.01 contrasted to the controls; ## P < 0.01 contrasted to the miRNA NC group.

Figure 3. Overexpression of miR-221-3p modifies Apoptosis and Cell Cycle in Propofol-Treated Astrocytes

The blank control, NC mimic, propofol + NC mimic, and propofol + miR-221-3p mimic groups were harvested for flow cytometry assay to measure the apoptosis (A) rate and cell cycle (B).

Figure 4. miR-221-3p regulates the TLR4–MyD88 Signaling Pathway in Propofol-Treated astrocytes
A. The blank control, NC mimic, propofol + NC mimic, and propofol + miR-221-3p mimic groups were harvested for qRT–PCR to quantify the mRNA levels of PCNA, GFAP, caspase-12, STAT3, GRP78, NF-κB, IL-6, IL-1β, TLR4, and MyD88. B. Western blotting was performed to detect the intracellular protein levels of TLR4, NF-κB (p50) and MyD88. ** P < 0.01 contrasted to the controls; ## P < 0.01 contrasted to the miRNA NC group * P < 0.05, ** P < 0.01 compared with the blank controls. * P < 0.05, ** P < 0.01 compared with the propofol group. * P < 0.05, ** P < 0.01 compared with the propofol + NC mimic group.

Figure 5. TLR4 is a Target of miR-221-3p

Dual-Luciferase Reporter Assay was performed to identify the association between TLR4 and miR-221-3p. * P < 0.05.

Figure 6. Knockdown of TLR4 Could Suppress the Apoptosis Rate in Propofol-Treated Astrocytes

A. Detection of knockdown efficiency of sitlr4 by qRT–PCR. B. Knockdown of TLR4 could suppress the apoptosis rate in propofol-treated astrocytes. C. The apoptosis rate in astrocytes in the group of siNC with propofol treatment. D. The apoptosis rate in astrocytes in the group of siTLR4 with propofol treatment. ** shows a very obvious difference in the blank controls (p <0.01), and ## shows a very obvious difference from the siNC group (p <0.01).
| Primer     | Sequence (5’-3’)                  |
|------------|-----------------------------------|
| PCNA-rF    | GCTTCGGCAGCACATATACTAAAT          |
| PCNA-rR    | CGCTTCACGAATTTCGCTGTCAT           |
| GFAP-rF    | GGTGTGGAGTGCGCTTCGTATAGG          |
| GFAP-rR    | GGACACATTTCAGTCATTTTCT            |
| TLR4-rF    | GATGCTCAGACATAGGCGAGTTTC          |
| TLR4-rR    | CACTCGAGGTAGGTGTTTCTGCTA          |
| NF-κB-rF   | GCACGGATGACAGAGGCGGTATAAGG        |
| NF-κB-rR   | GGCGGATGATCTCCTTCTCTGCTG          |
| IL-6-rF    | AAGAAAGACAAAGGCCAGAGTC            |
| IL-6-rR    | CACAAACTGTATGCTTTAGGA            |
| IL-1β-rF   | AGGCTGACAGACCCCAAAGAT             |
| IL-1β-rR   | CTCCACGGGCAAGACATAGGTAA           |
| MyD88-rF   | TGGTGGTGTTCGCTGACGAT             |
| MyD88-rR   | GATCAGTCGCTTCTGTTGGA             |
| caspase-3-rF | GTATTGAGACAGACAGTGG              |
| caspase-3-rR | CACGGATGATCTTCTTGTG              |
| caspase-12-rF | GGAAGGTAGGCAAGAGT               |
| caspase-12-rR | GTGAGTAGCTGCTGACAT              |
| MyD88-rF   | GCTGACTTGGAGCCTGATTCT            |
| MyD88-rR   | ATGGGTTGCTGGGAGTAA               |
| STAT3-rF   | CACCTTGATTGAGGTCAAGAC            |
| STAT3-rR   | AGGAATCGGCTATATTGCTGGT           |
| GRP78-rF   | TCAGCCACCGTAACAAT                |
| GRP78-rR   | CAAACTTCTCGGCGTCAT               |
| rno-miR-221-3p-RT | GTCGTATCCAGTGACGGGTCGAGGTATTCGCA |
|            | CTGGATACGACGAAACC                |
| rno-miR-221-3p-F  | GCACGGTACATATTTGCTGCTG          |
| rno-U6-RT  | CGCTTCAGGAAATTTCGCTGTCAT         |
| rno-U6-F   | GCTTCCAGCAGCAATATATATACAAAAT     |
| rno-U6-R   | CGCTTCAGGAATTTTCGCTGTCAT         |
| GAPDH-rF   | AGACAGCCGACTTTCTTGT              |
| GAPDH-rR   | CTTGGCGTGGTAGAGTCAT              |
A hsa-miR-221-3p 3' CUUUGGGUCGUCUGUUAACAUUCG 5'
TLR4 3'UTR-WT 5'...GGGAAUGGAAAUUGGUAGCC...3'
TLR4 3'UTR-MUT 5'...GGGAAUGGAAAUUGGAACUCGC...3'

B

|                | Relative luciferase activity |
|----------------|------------------------------|
| pGL3-control   | 8                            |
| pGL3-TLR4 3'UTR WT | 6                            |

NC mimics
miR-221-3p mimics

**
