ORIGINAL RESEARCH

Long-Term Alcohol Exposure Aggravates Ischemic Stroke-Induced Damage by Promoting Pericyte NLRP3 Inflammasome Activation via Pre-Activating the TLR4/NF-κB Pathway in Rats

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Background: Ischemic stroke (IS) is one of the leading causes of death and disability in the world, and alcohol consumption has been gaining attention as an independent risk factor for IS. Blood-brain barrier (BBB) dysfunction and neuroinflammation are the core of cerebral ischemia/reperfusion (I/R) injury, and pericytes play a crucial role in the structure and function. This study is to explore the effects of long-term alcohol consumption on IS and the potential mechanisms of pericytes.

Methods: Rat models of long-term alcohol intake followed by transient middle cerebral artery occlusion stroke (EtOH+tMCAO) and cell models of oxygen-glucose deprivation/reoxygenation (OGD/R) with alcohol pre-treatment were constructed.

Results: Worsened infarct volume, neurological scores, and BBB disruption were observed in the EtOH+tMCAO group compared with the tMCAO group, and immunofluorescence staining showed increased pericytes NLPR3 inflammasome activation at the ischemic penumbra. In vitro, pericyte mortality and LDH release elevated pre-treated by alcohol after OGD/R, and amplified expression of NLRP3 inflammasome was detected by Western blotting and qPCR. Alcohol pre-treatment activated the TLR4/NF-κB pathway, and transfecting pericytes with TLR4-small interfering RNA (siRNA) to block TLR4 signaling markedly restrained NLRP3 inflammasome over-activation. Injecting TAK-242 in rats alleviated neurological impairment caused by alcohol.

Conclusion: Long-term alcohol pre-treatment aggravated ischemic stroke-induced brain damage by activating NLRP3 inflammasome via TLR4/NF-κB signaling pathway in the pericytes.

Keywords: cerebral ischemia, chronic ethanol exposure, pericytes, TLR4, NLRP3 inflammasome

Introduction

Stroke is the most common cerebrovascular disease.[1] In neuroscience, ischemic stroke (IS) is an urgent concern due to its increasing health and economic burden.[2] However, the pathogenesis of IS remains unclear. Ischemia triggers a cascade of events, including Ca^{2+} influx, glutamate efflux, and free radicals release, leading to cell death and secretion of inflammatory cytokines. Resident immunological brain cells involved in inflammatory responses are activated. With the disruption of the blood-brain barrier (BBB), activated blood-borne immune cells accumulate from the periphery, secreting a plethora of inflammatory cytokines.[3] Pro-inflammatory cytokines and chemokines promote leukocyte infiltration in a feed-forward manner and exacerbating the BBB damage.[4] This inflammatory cascade produces more inflammatory intermediaries, such as the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLPR3) inflammasome, which causes cell pyroptosis, increases BBB damage, and promotes neuronal death.[5]

Alcohol, also referred to as ethanol, is the most abused substance in the world.[6] Many investigations have reported that chronic alcohol consumption is an independent risk factor for IS in men.[7] Widely accepted epidemiological research has revealed that the impact of alcohol shadow on stroke depends on the quantity consumed and time; however, the relationship between alcohol consumption and the incidence and prognosis of IS stays controversial.[8–10] Therefore, a comprehensive and systematic study of the consequences of alcohol intake on cerebral infarction and its underlying mechanism is necessary.

Pericytes recreate a substantial position in conserving the integrity of the BBB and function in regulating blood flow, cerebral vasculature formation, and neuroinflammation.[11] Accumulating evidence indicates that pericytes are essential for IS progression.[12] In a transient MCAO (tMCAO) model, pericyte-deficient mice have increased cerebral edema after cerebral infarction compared to wild mice in the tMCAO model. In addition, pericyte-derived matrix metalloproteinases (MMPs) cause secondary lysis of tightly junctioned structures in IS. Pericytes are involved in leukocyte recruitment by expressing adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β acting on pericytes stimulate them to increase the expression of ICAM-1 and VCAM-1 and recruit peripheral immune cell infiltration.[13] Upon stimulation, pericytes have neuroinflammatory effects which can lead to disruption of the BBB.[14] However, whether pericytes participate in alcohol-induced stroke pathological processes and the specific underlying mechanisms remain unclear.

Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are pattern recognition receptors that play important roles in initiating immune response,[15] reportedly contributing to the pathological progression of IS-induced cerebral injury. NLRP3 inflammasome comprises NLRP3, apoptosis-associated speckle-like protein containing a C-terminal caspase recruitment domain (ASC), and the effector protein, pro-caspase-1. After stimulation, NLRP3 recruits ASC through domain interactions and activates pro-caspase-1. Activated caspase-1 activates pro-interleukin (IL)-1β and pro-IL-18 to their mature (IL-1β and IL-18) forms, which mediate inflammatory responses and initiate inflammatory cell death, scilicet pyroptosis.[16] Current research indicates that NLRP3 mRNA was expressed in untreated pericytes, and an increased expression of pyroptosis-associated proteins was detected following hypoxia.[17] TLRs are considered major factors of alcohol-induced neuroimmune activation. Autopsy of alcoholic brain tissues revealed raised TLR2, TLR3, and TLR4 expression, which correlates with the loss of BBB integrity;[18] however, alcohol-induced BBB disruption is not evident in the brain tissues of TLR4-KO mice.[19] In vitro, alcohol-induced TLR4 activation was observed in microglia and astrocytes, which release inflammatory mediators.[20,21] However, whether alcohol activates the TLR4/NF-κB signaling pathway in pericytes is yet to be investigated. Additionally, certain investigations have revealed that TLR4 promotes the transcription of NLRP3 components by triggering the TLR4/NF-κB signaling pathway.[22] The relationship between alcohol consumption, NLRP3 inflammasome activation in the pericytes, and stroke needs elucidation.

Therefore, we investigated the activation of NLPR3 inflammasome and TLR4/NF-κB pathway in pericytes in the ischemia-reperfusion (I/R) injury combined with alcohol treatment, both in vivo and in vitro, to observe the effects of long-term alcohol consumption on ischemic brain injury in rats and the possible inflammatory pathways and potential regulatory mechanisms of pericytes plays.

Methods
Animals
Sprague-Dawley (SD) rats (150±25 g, specific pathogen-free grade) were purchased from and bred at the Henan Provincial Laboratory Animal Center in Zhengzhou, China. All animal experiments are approved by the Animal Ethics Committee of Henan Provincial Laboratory Animal Center (approval number: ZZU-LAC20201225[03]), and the guidelines followed for the welfare of laboratory animals are the National Standard of the People's Republic of China General Rules for the Welfare of Laboratory Animals (GB/T 42011–2022). The experimental groups included the NC (normal control) group, EtOH (ethanol intake) group, tMCAO (transient middle cerebral artery occlusion) group, EtOH +tMCAO group, LPS (Lipopolysaccharides)+tMCAO group, EtOH+TAK-242+tMCAO group.

Based on the experimental protocol, the rats were intraperitoneally injected with LPS (L3024, Sigma-Aldrich, USA) solution or TLR4 inhibitor TAK-242 (Sigma-Aldrich, USA, 614,316), followed by sham surgery or tMCAO. The rats in the non-drinking group were injected with LPS solution (1 mg/kg) or an equal volume of saline 4 h before tMCAO.

TAK-242 (3 mg/kg) was dissolved in a 5% dimethyl sulfoxide (DMSO) solution and was administered to the rats once daily during the 48 d of alcohol consumption, and an equivalent amount of DMSO was used as control.

Chronic Alcohol Intake Model

Following the rats' adaptation to the environment, they were weighed to obtain their basal weight. Initially, four 50mL water bottles were placed in the housing cage, with three of the bottles replaced with a 3% (v/v) ethanol solution for four days. Thereafter, the ethanol concentration was gradually increased to 6–10% for four days each. Finally, all bottles reached a concentration of 20% ethanol and were maintained for another five weeks. The volume of the remaining fluid was measured daily. The control group received water for 48 days. The tMCAO or sham surgery was performed on day 49.

tMCAO Model

The tMCAO rat model was constructed as previously described.[23] Briefly, following anesthesia with 10% isoflurane, make a median incision in the rat's neck and carefully isolate the right external carotid artery. Insert a tailor-made filament (Beijing Cinontech Co. Ltd.;, China, 2838-A4) into the internal carotid artery from the incision of the common carotid artery to block the beginning of the middle cerebral artery. The end of the filament was pulled outward for 1 cm to initiate reperfusion after a 2 h obstruction. Sham surgery was performed as a control, including the same operations as the tMCAO but without inserting the filament and subsequent reperfusion. A homoeothermic heating pad was used until the rats regained consciousness. Rats that matched behavioral evaluation were deemed successful models for subsequent experiments. Rats with subarachnoid hemorrhages were excluded.

Infarct Volume and Brain Edema Measurements

After 24 h post-modeling, brains were sliced to an approximately 2-mm thickness and stained with 2, 3, −5-triphenyl tetrazole (TTC) solution (Servicebio, Wuhan, China, G3005) at 37 °C for 30 min. Images were captured using a camera, and ImageJ software was used to analyze the infarct size. The total infarct size was expressed as the sum of the damaged area of the six sections, as a ratio to the sum area of the non-ischemic hemisphere, to correct the effect of edema in the infarct hemisphere on the area. The wet-dry method was used to measure cerebral edema. Fresh brains are weighed to wet weight and then dried thoroughly to obtain dry weight.

Behavioral Tests

Neurological deficits were evaluated using Longa's test 24 h after tMCAO. Longa's score criteria: 0 points, no defect; 1 point, the contralateral forelimb cannot be fully straightened; 2 points, rotation to the paralyzed side while waking; 3 points, dumping to the paralyzed side while waking; 4 points, unable to walk spontaneously or unconscious. The open-field test was used to assess locomotive ability. Placed each animal in a square box (100 × 100×50 cm) for 2 min to explore the apparatus freely after pre-training. The movement distance and resting time were recorded and analyzed using Smart v3.0. The rotarod experiment was conducted to evaluate the rats' motor coordination declination. The rats were trained to adapt to the rotating rod apparatus for 3 days before tMCAO induction. During the test, the duration of the rat's crawling on the rotating rod and falling off numbers in 2 min were recorded. Data from more than three replications.

Immunofluorescence Staining and TUNEL Staining

Rats got cardiac perfusion with saline and 4% paraformaldehyde after anesthesia. Followed by sucrose gradient dehydration, the brains were quickly frozen, and a layer of OCT glue was applied. Subsequently, 10 μm coronal section slices were dried for 1 hour, washed twice, and blocked with 5% bovine serum albumin (BSA) for 30 min. Add the prepared primary antibodies and incubate at 4 °C overnight. Antibodies of PDGFR-β (mouse, Cell Signaling Technology, US, 3175S), NLRP3 (rabbit, Abcam, US, ab263899) and IL-1β (rabbit, bioss, China, bs-0812R) were diluted in 1:100 with PBS. After washing thrice with PBS, the sections were incubated with a mixture of goat anti-mouse Alexa Fluor 488-labeled IgG and goat anti-rat Alexa Fluor 555-labeled IgG (Beyotime, China, 1:500) for 1 h. The sections were washed and treated with antifade mounting medium containing DAPI and sealed with a cover glass slide. TUNEL BrightGreen Apoptosis Detection Kit (Vazyme Biotech, China, A112-03) was used

for TUNEL staining. Endothelial cells (ECs) were incubated with CD31 antibody (rabbit, Novus, USA, AF3628-SP) and stained in red by donkey anti-rabbit Alexa Fluor 555-labeled IgG (Beyotime, China, 1:500). Ischemic penumbra areas were observed and photographed using an ultra-high-resolution laser confocal microscope (LSM880, Carl Zeiss, Germany).

Pericyte Primary Culture

The brain microvascular pericytes were isolated from rats aged three weeks. After disinfection, the brains were fetched out in a standard sterile cabinet, and quickly placed in pre-cooled sterile phosphate buffer saline (PBS). Removing the pia mater, white matter, and large blood vessels from the cerebral cortex, the brain tissue was cut into $1mm^3$ by ophthalmic scissors and placed into a 50mL centrifuge tube containing Dulbecco-modified Eagle's medium (DMEM). Collagenase type 2 (1 mg/mL, Sigma, USA) and DNase I (15 g/mL, Sigma, USA) were added to the DMEM, and shaking digestion lasted at 37°C for 1.5 h. Centrifuge 1000×g for 8 min, remove the supernatant, add 20% BSA-DMEM, mix well, centrifuge (1000×g, 20 min), adsorption, and remove neurons and myelin sheath. After discarding the supernatant, DMEM containing collagenase/dispersase (10 mg/mL, Sigma, USA) and DNase I (1 mg/mL) was added for re-suspension and digestion at 37°C for 1h. After adding 10 mL of DMEM and centrifuging at 700×g for 6 min, we obtained isolated microvascular fragments, which were yellowish-white precipitates. Suspending with the culture medium containing 10% FBS and 1% penicillin-streptomycin in DMEM, place the cells in a 37°C incubator with 5% CO2.

OGD/R and Drug Treatment

To mimic I/R insult in vitro, the cells were washed with PBS, and the culture medium was changed to glucose-free DMEM. The cells were then incubated in the Tri-gas Incubator chamber (Eppendorf Galaxy 48R) under hypoxic conditions (37°C, 1% O_2, 5% CO_2, and 94% N_2) for 4 h. Then the pericytes were incubated for 24 h back in the normal gas culture medium. Purified pericytes were treated with ethanol or LPS (L3024, Sigma-Aldrich, USA). Sterile ethanol was added to the EtOH / EtOH + OGD/R group to obtain the final concentration of 50mM, which is the range of blood alcohol concentrations in chronic drinkers. To avoid alcohol volatilization, the plates were placed in a closed plastic box containing a similar concentration of alcohol solution and injected with 5% CO_2. In the control group, an equal volume of PBS instead of ethanol. The LPS+OGD/R group was administered an optimal dose of LPS solution (PBS configuration) to obtain the concentration of 50 ng/mL for 4 h before OGD/R treatment.

Flow Cytometry

After OGD/R or ethanol treatment, pericytes were dissociated with trypsin. Apoptosis detection was performed using the Annexin-V/PI Cell Apoptosis Detection Kit (KGA108, KeyGEN BioTECH, China) according to the instructions. The apoptotic rates were detected using flow cytometry (LSRFortessa, BD Biosciences, USA).

Lactate Dehydrogenase (LDH) Release Assay

After the indicated treatment, the supernatants of the pericytes cultures were collected and LDH release was measured by using an LDH Cytotoxicity Assay Kit (Beyotime, China) to detect the pyroptotic cell death.

Supernatant Cytokine Measurement

Enzyme-linked immunosorbent assay (ELISA) was used to detect the cytokine concentrations in the supernatant of pericytes. The level of IL-1β and IL-18 cytokines were measured using ELISA Kits (Elabscience, China).

Real-Time Quantitative PCR

An extraction of total RNA from tissue and cells was performed with the TRIzol® reagent (Invitrogen/Life Technologies, USA). Real-time quantitative PCR (RT-qPCR) was performed using a QuautStudio-5 (Thermo Scientific, USA) instrument and SYBR Green Pro Taq HS Premix qPCR Kit IV (AG11746, Accurate Biology), following the instructions for system preparation and reaction time. The mRNA level was normalized to GAPDH level, and the relative quantity was

calculated using the threshold cycle (Ct) = 2-ΔΔCt of the concerning target amplification. The sequences of each specific primer (Table 1), designed by the Servicebio Institute of Biology (Wuhan, China).

Western Blotting

Proteins in homogenized brain ischemic penumbra tissues or cells were extracted using RIPA lysis buffer (Solarbio, China) containing phenylmethylsulfonyl fluoride. Protein concentration was quantified according to the BCA Protein Assay Kit (Solarbio, China). After adding loading buffer and boiling at 95°C, equal amounts of sample proteins were separated on a 10% sodium dodecyl-polyacrylamide gel and subsequently transferred to polyvinylidene difluoride membranes. Blocked with 5% skim milk and incubated the membranes with primary antibodies overnight at 4°C. Antibodies of MMP9 (rabbit, Proteintech, China,10375-2-AP), Occludin (rabbit, Proteintech, China, 27260-1-AP), TLR4 (rabbit, Abcam, US, ab217274), NLRP3 (rabbit, Abcam, US, ab263899), GSDMD (rabbit, Abcam, US, ab219800), ASC (rabbit, Cell Signaling Technology, US, 67824S), caspase-1 (rabbit, Abcam, US, ab207802), NF-κ b p65 (rabbit, bioss, China, bs-20160R), phospho-NF-κ b p65 (rabbit, bioss, China, bs-3485R), IL-18 (rabbit, bioss, China, bs-42148R), IL-1β (rabbit, bioss, China, bs-0812R), and GAPDH (rabbit, Cell Signaling Technology, USA, #2118S) were diluted in 1:1000 with PBS. The membranes were washed and incubated with a HRP conjugated goat anti-rabbit IgG h+l antibody antibody (absin, China, 9048–46-8) for 1 h, and detected using an enhanced chemiluminescence substrate solution. Gray areas were analyzed using ImageJ software.

siRNA Transfection

Primary pericytes were transfected with rat TLR4-siRNA (forward, 5'-dTdT-3'; reverse, 3'-dTd-5') or NC-siRNA (RiboBio. Co. LTD, China) according to the kit's instructions. After 24 h of transfection, total cell RNA or protein was extracted, and RT-qPCR of TLR4 was performed to examine the effect of silencing. siRNA-transfected pericytes were treated with EtOH, followed by OGD/R treatment.

Statistical Analysis

The data were tested for normality and were found to conform to a normal distribution and expressed as mean ± SEM. Statistical analysis was conducted using t-test or one-way ANOVA, followed by the Bonferroni multiple comparison with the software GraphPad Prism version 9 (GraphPad Software, USA). It was defined statistically significant when $P < 0.05$. Each experiment is repeated independently at least three times. The exact sample sizes (n numbers) are provided in the figure legends.

Results

Chronic Alcohol Intake Increases Infarct Volume and Aggravates Functional Deficits in I/R Injury

This study constructed tMCAO models and used TTC staining to assess the area of cerebral infarction in each group. First, excluding the unsuccessful models, surviving rats were sacrificed 24 hours after reperfusion, and the survival rate was lower in the EtOH+tMCAO group than in the tMCAO group (Supplementary Figure 1). As shown in Figure 1A, the brain tissues of the rats in the sham-operation and EtOH groups were stained red, and unstained infarct areas appeared in rats that got tMCAO operation. The infarct area of rats in the EtOH+tMCAO group was significantly larger than that in the tMCAO group (P=0.0023) (Figure 1B). Neurological scoring results showed significant neurological deficits in the tMCAO group compared to the control and alcohol treatment worsened neurological deficits following tMCAO, with

Table 1 Sequences of Primers Used for RT-qPCR (Rat Species)

| Gene	Primer Sequence (Forward)	Primer Sequence (Reverse)
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
TLR4	GATCTGAGCTTCAACCCCCT	TTGTCTCAATTTCACACCTGGA
NLRP3	CTGCAGAGCCTACAGTTGGG	GTCCTGCTTCCACACCTACC
Pro-IL-1β	AGGATCAGCAAGCTACGACA	TTGTCAACGATCCAGACTCTC
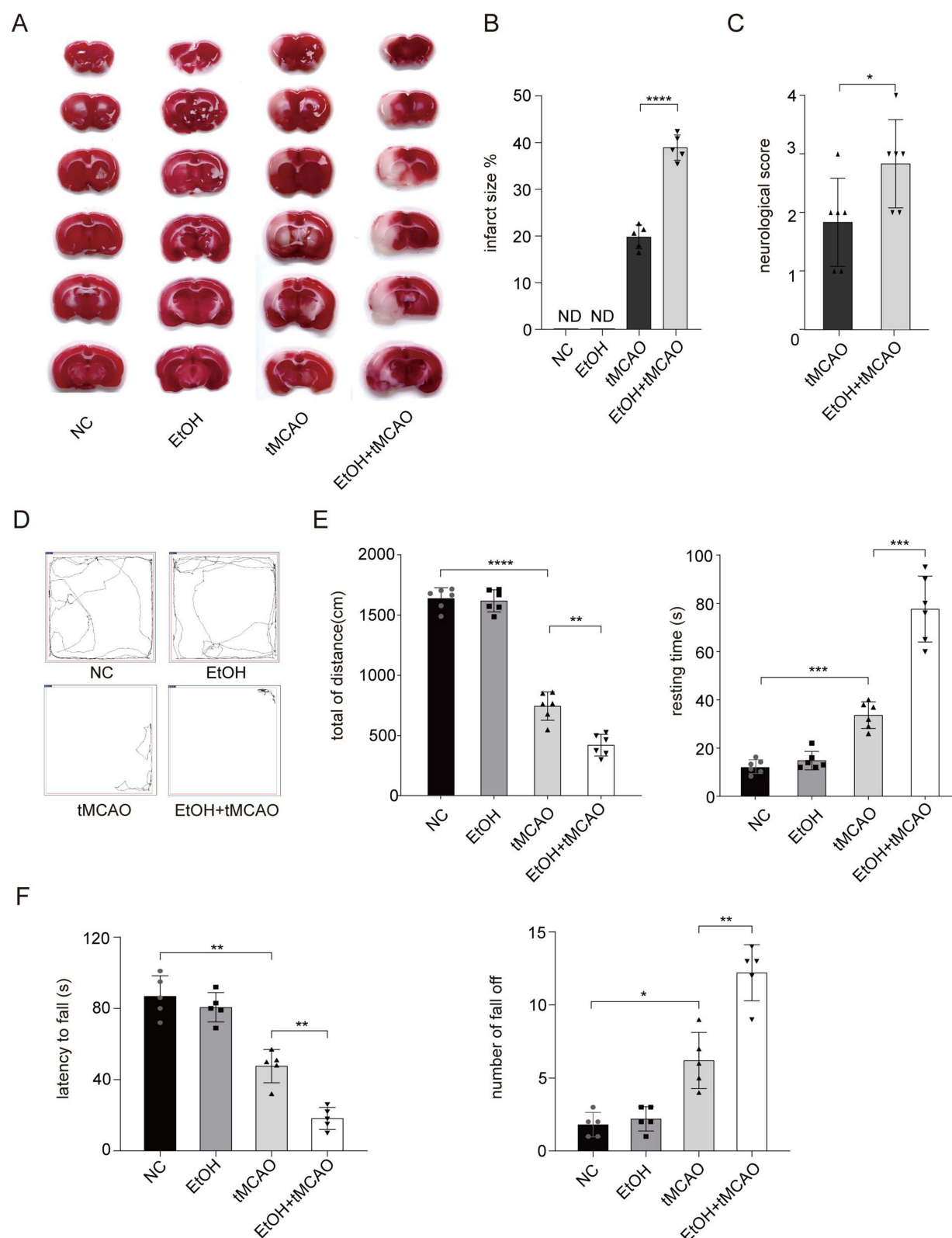
Figure 1 Alcohol aggravates cerebral I/R injury in rats. (**A**) TTC staining was used to detect infarct size. The infarct area is white. (**B**) The quantitative analysis of average infarct size (n = 5). (**C**) Neurological scores of each group (n = 6). (**D**) Trajectories of rats in the open-field test. (**E**) Statistic bar graph of the total moving distance (cm) in 2 min and the resting time (s) in the open field test (n = 6). (**F**) Statistic bar graph of the holding time on the rotating bar (s) and the fall-off numbers in the rotarod test (n = 5). ND means "not detected", *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001.

a higher Longa score (P=0.0442) (Figure 1C), a shorter movement distance (P=0.0025), a reduced movement time (P=0.0009) in the open-field experiment (Figure 1D and E), a shorter retention time (P=0.0033), and an increasing number of falls off (P =0.0061) in the rotarod experiment (Figure 1F).

Chronic Alcohol Intake Aggravates Brain Edema and Decreases BBB Junction Integrity in I/R Injury

The rats' brain water content in the EtOH group did not show any observable differences compared with the control group (P=0.992). Significant brain edema was observed in the tMCAO group 24 h after reperfusion compared to the control (P=0.0004), and the water content was higher in the EtOH+tMCAO group (P=0.0007) (Figure 2A). TUNEL
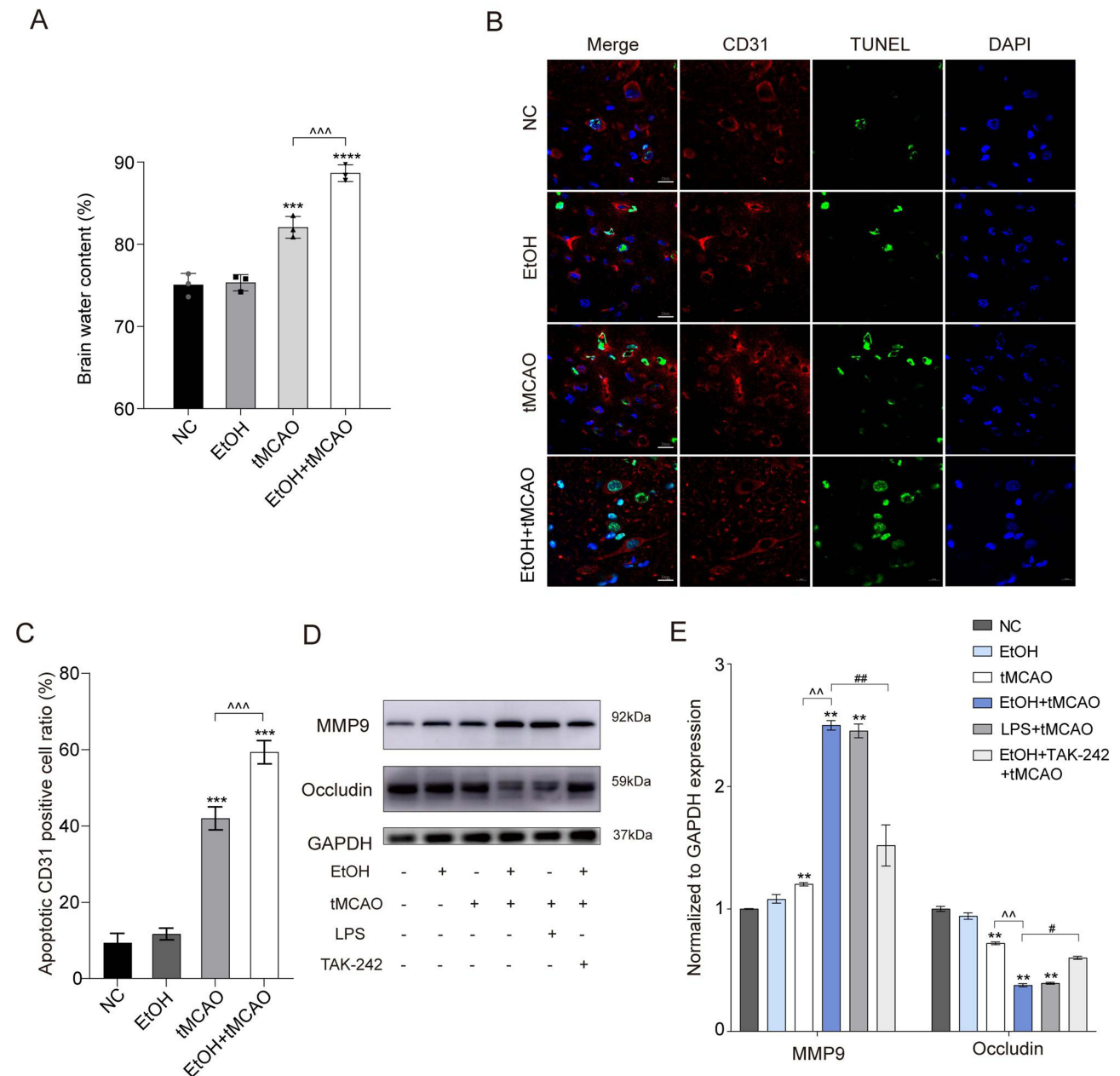
Figure 2 Chronic alcohol intake aggravates BBB dysfunction of I/R injury in rats. (**A**) Average brain water contents (%) measured by wet-dry method (n = 3). (**B**) Representative images of TUNEL staining (green) and CD31 (red) in each group (n = 3). Nuclei were stained DAPI (blue). Scale bar = 10 μm. (**C**) The quantitative analysis of the apoptotic endothelial cells ratio in different groups (n = 3). (**D** and **E**) WB detection of MMP-9 and Occludin proteins in the ischemic penumbra cortex in different groups. *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001 versus control, ^p < 0.05, ^^ p < 0.01, ^^^p<0.001 versus tMCAO, #p < 0.05, ##p < 0.01 versus EtOH+tMCAO.

staining showed an increase in apoptotic CD31$^+$ endothelial cells in the ischemic penumbra cortex compared with the control group (P=0.0006). The EtOH+tMCAO group showed an increased ratio of apoptotic ECs compared to the tMCAO group (P=0.0001) (Figure 2B and C). Western blotting (WB) analysis showed that EtOH intake aggravated occludin protein reduction in the ischemic penumbra cortex, which is a main tight junction protein associated with BBB permeability[24] and decreased in the tMCAO group (P=0.0039). Furthermore, we measured the level of matrix metallo-proteinase-9 (MMP-9), which can degrade endothelial tight junctions,[25] was found upgraded in the tMCAO group (P=0.0008), and the MMP-9 levels were higher in the EtOH+tMCAO group (P=0.0003) (Figure 2D and E). These findings indicated that alcohol increases BBB dysfunction following IS.

Chronic Alcohol Intake Upregulated NLRP3 Inflammasome Expression in the Pericytes After I/R Injury

Pericytes are structurally adjacent to ECs and functionally impacted BBB. To investigate the neuroinflammation role of pericytes playing in the increased apoptosis of ECs, immunofluorescence staining of PDGFR-β (marker of pericyte) and NLRP3 inflammasomes was performed. The results showed increased expression of the key molecules, NLRP3 and IL-1β in the pericytes at the ischemic penumbra area (P=0.0053 and 0.0002, respectively), and the ischemic penumbra area of chronic alcohol intake group showed higher activation of these proteins (P=0.0109 and 0.0008, respectively) (Figure 3A-D). However, the results showed no observable differences in NLRP3 inflammasome activation in the pericytes in the EtOH group compared to the control.

## Chronic Alcohol Intake Activates TLR4 in the Pericytes

TLR4 signaling pathway could interact with NLRP3 inflammation activation pathway, and TLR4 is reported to be an important target that alcohol affects in the central nervous system. Therefore, immunofluorescence staining was conducted, and the results showed TLR4 protein expression in the pericytes was upregulated in both alcohol-intake rats (EtOH group and EtOH+tMCAO group) (P=0.0007 and 0.0003, respectively). TLR4 expression was also elevated in the ischemic penumbra region in the tMCAO group (P=0.0031) but with much weaker fluorescence intensity than the other two groups (Figure 4A and B).

Alcohol Increases Pericyte Mortality and Leakage After OGD/R via TLR4 in vitro

To further validate our findings, primary pericytes were isolated from the juvenile rats' cerebral cortex and characterized by immunostaining with the cell surface marker PDGFR-β (Figure 5A). We conducted OGD/R experiments to mimic the I/S injury in vitro. Detecting cellular mortality by flow cytometry, we found that EtOH treatment resulted in significantly higher pericyte death rates caused by OGD/R (P=0.0318) (Figure 5B and C). We also explored the extent of cellular leakage by LDH release assays, due to perforation of the plasma membrane during cellular pyroptosis. Ethanol treatment upregulated LDH release of pericytes in OGD/R (P=0.0036). These results suggest that pericytes are more vulnerable to OGD/R injury and more cellular pyroptosis occurs after alcohol treatment (Figure 5D).

To verify that alcohol is active TLR4 and impacting NLRP3 activation in pericytes, we transfected TLR4-siRNA to block TLR4 activation, followed by alcohol treatment. TLR4 mRNA expression was efficiently inhibited by TLR4 siRNA2 (P<0.0001) (Figure 5E), as presented in Table 2. The WB results confirmed that TLR4 protein expression is increased in OGD/R followed by EtOH treatment (P=0.0061), which inhibited by TLR4 siRNA (P=0.0034) but not by negative control siRNA (NC siRNA) (P=0.7099) (Figure 5F and G). Furthermore, we used LPS, the specific agonist of TLR4 signaling, to treat pericytes before OGD/R, and observed a similar tendency of alcohol's effection, that cell mortality (P=0.0164) and LDH release increased (P=0.0003). Compared with EtOH+OGD/R group, pericyte mortality (P=0.012) and LDH release in TLR4 siRNA+EtOH+OGD/R group were decreased (P=0.0013) (Figure 5H-J). The optimal concentration of LPS was determined by both CCK-8 and RT-qPCR experiment (Supplementary Figure 2).
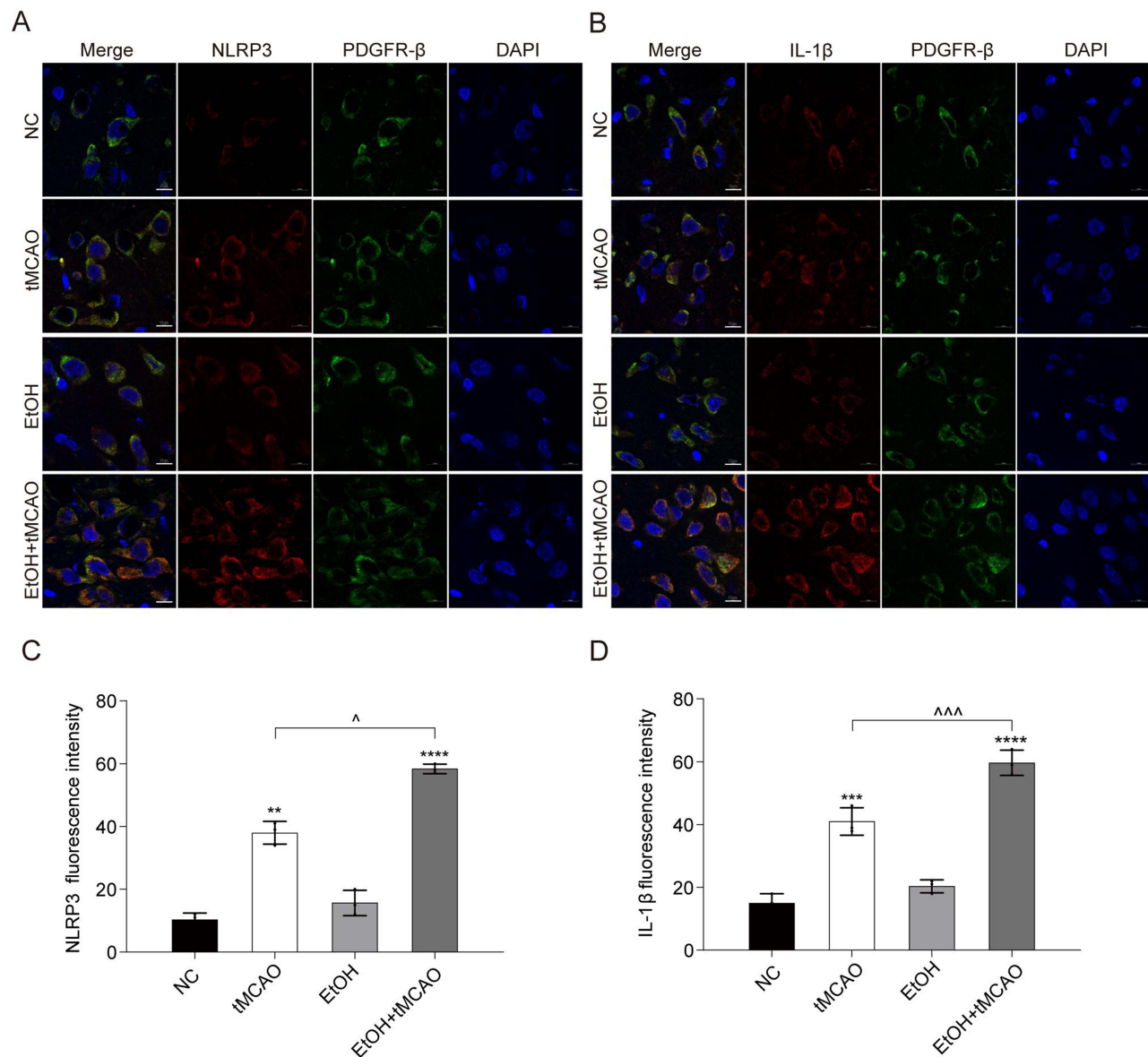
Figure 3 Chronic alcohol intake upregulated NLRP3 inflammasome expression in the pericytes after I/R injury. (**A** and **B**) Representative immunofluorescence images showing NLRP3 (red) or IL-1β (red) in pericytes (PDGFR-β: green) in each group (n = 3–5 rats for each group). Nuclei were stained with DAPI (blue). Scale bar = 10 μm. (**C** and **D**) Semiquantitative analysis of average NLRP3 or IL-1β fluorescence intensity in each group. *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001 versus control, ^p < 0.05, ^^ p < 0.01, ^^^p<0.001 versus tMCAO.

Alcohol Activate TLR4/NF-κB Signal Pathway and Increases NLRP3 Inflammasome Activation After OGD/R in vitro

A series of WB was performed to examine the TLR4/NF-κB signaling pathway and NLRP3 inflammation proteins under different stimulation conditions. The results showed that after alcohol treatment, TLR4 (P=0.0008) and its downstream target, phospho-NF-κB (p-NF-κB) (P=0.0067), were elevated in the pericytes, accompanied by more NF-κB translocated into the nucleus (Figure 6A and B), demonstrating the activation of TLR4/NF-κB signaling pathway. No significant increase in NLRP3 (P=0.323) was observed in the EtOH-treated group. We conducted OGD/R in both alcohol-treated and untreated groups. The results demonstrated that the expression of NLRP3 (P=0.0497), ASC (P=0.0278), full and cleaved GSDMD (P=0.0021 and 0.0206), pro and cleaved caspase-1 (P=0.0247 and 0.0003), pro-IL-18 (P=0.0134), IL-18 (P=0.0473), pro-IL-1β(P=0.003) and IL-1β (P=0.003) in the pericytes elevated following OGD/R, with a more pronounced increase observed in the EtOH+OGD/R group (all P < 0.05) (Figure 6C-E). These findings validate the
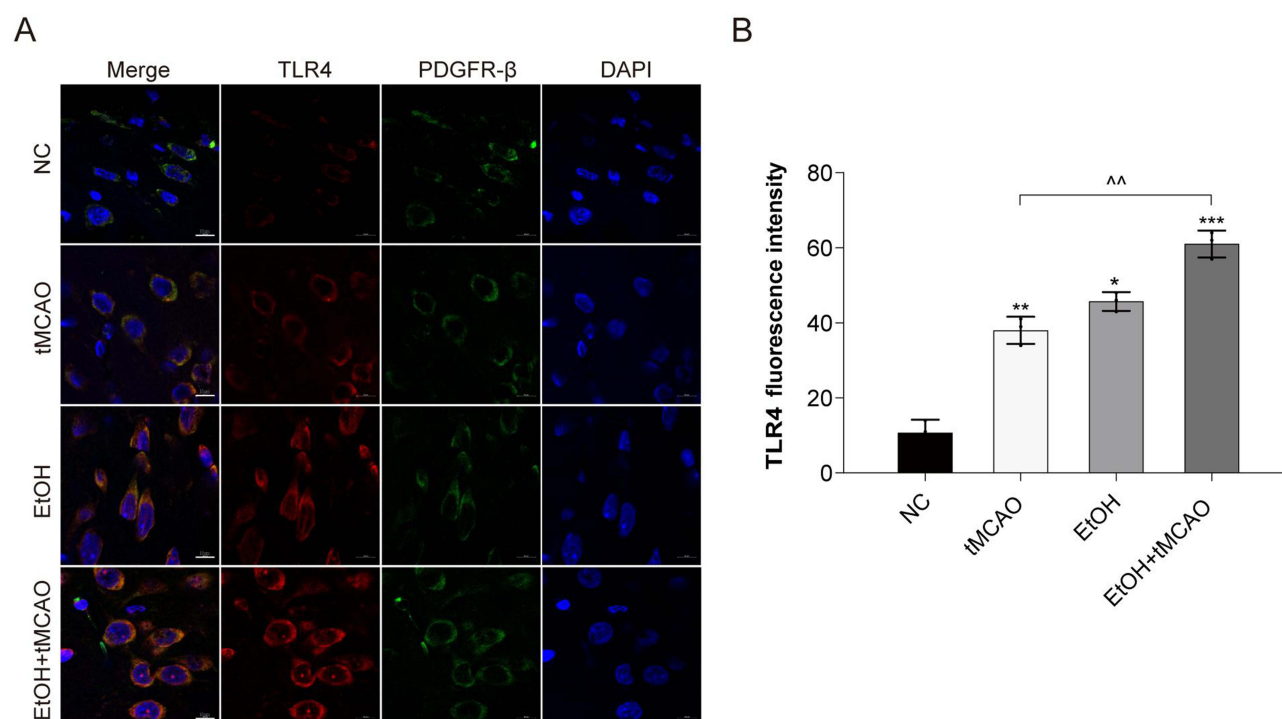
Figure 4 Chronic alcohol intake activates TLR4 in the pericytes. (**A and B**) Representative immunofluorescence images and semiquantitative analysis of TLR4 (red) in pericytes (PDGFR-β: green) in each group (n = 3–5 rats for each group). Nuclei were stained with DAPI (blue). Scale bar = 10 μm. *p < 0.05, ** p < 0.01, ***p<0.001 versus control, ^p < 0.05, ^^ p < 0.01 versus tMCAO.

suitability of the in vitro model as a representative of in vivo I/R injury. Furthermore, they confirm that alcohol treatment significantly enhances the upregulation of NLRP3 inflammasome-associated proteins in the pericytes following reperfusion, in line with the in vivo experiment results.

Using LPS to activate the TLR4 pathway, NLRP3-associated proteins upregulated in the LPS+OGD/R group compared to the OGD/R group (all P < 0.05), and the LPS+OGD/R group showed no observed differences with the EtOH+OGD/R group. As shown in the EtOH+OGD/R+TLR4 siRNA group, transfecting pericytes with TLR4 siRNA to block TLR4 signaling markedly weakened NLRP3 inflammasome activation in OGD/R with alcohol co-treatment (all P < 0.05) (Figure 6C-E). These results indicate that TLR4 is essential for NLRP3 inflammasome activation in the pericyte during cerebral I/R injury.

Alcohol Pre-Treatment Elevates NLRP3 and Pro-IL-1β mRNA Levels

Moreover, a series of RT-qPCRs was conducted. The results showed that OGD/R treatment increased NLRP3 (P=0.0375) and pro-IL-1β (P=0.0002) mRNAs in pericytes, and the mRNA levels were even higher in the EtOH+OGD/R group (P<0.0001 and 0.0065, respectively), consistent with the WB results. However, in the EtOH group, there was a significant elevation in TLR4 (P=0.0029) mRNA levels, accompanied by a notable increase in NLRP3 (P=0.0069) and pro-IL-1β (P=0.0084) mRNA levels (Figure 7A), which not observed in WB. This indicates alcohol treatment activated TLR4, and also promoted the transcription of the NLRP3 inflammasome gene. However, no increase in NLRP3 functional proteins was detected in WB. Whereas, elevated mRNA levels can be a prerequisite for the explosively amplified activation of the NLRP3 inflammasome after OGD/R.

Furthermore, the NC siRNA was employed as a negative control. The NLRP3 inflammasome-associated proteins (NLRP3, caspase-1, IL-18, and IL-1β) demonstrated no significant difference in the EtOH+OGD/R+NC siRNA group in comparison to that observed in the EtOH+OGD/R group (all P>0.05). This ruled out the possibility of pyroptosis-associated protein alterations resulting from the synthesis method and process of siRNA, as well as transfection reagents (Figure 7B and C).
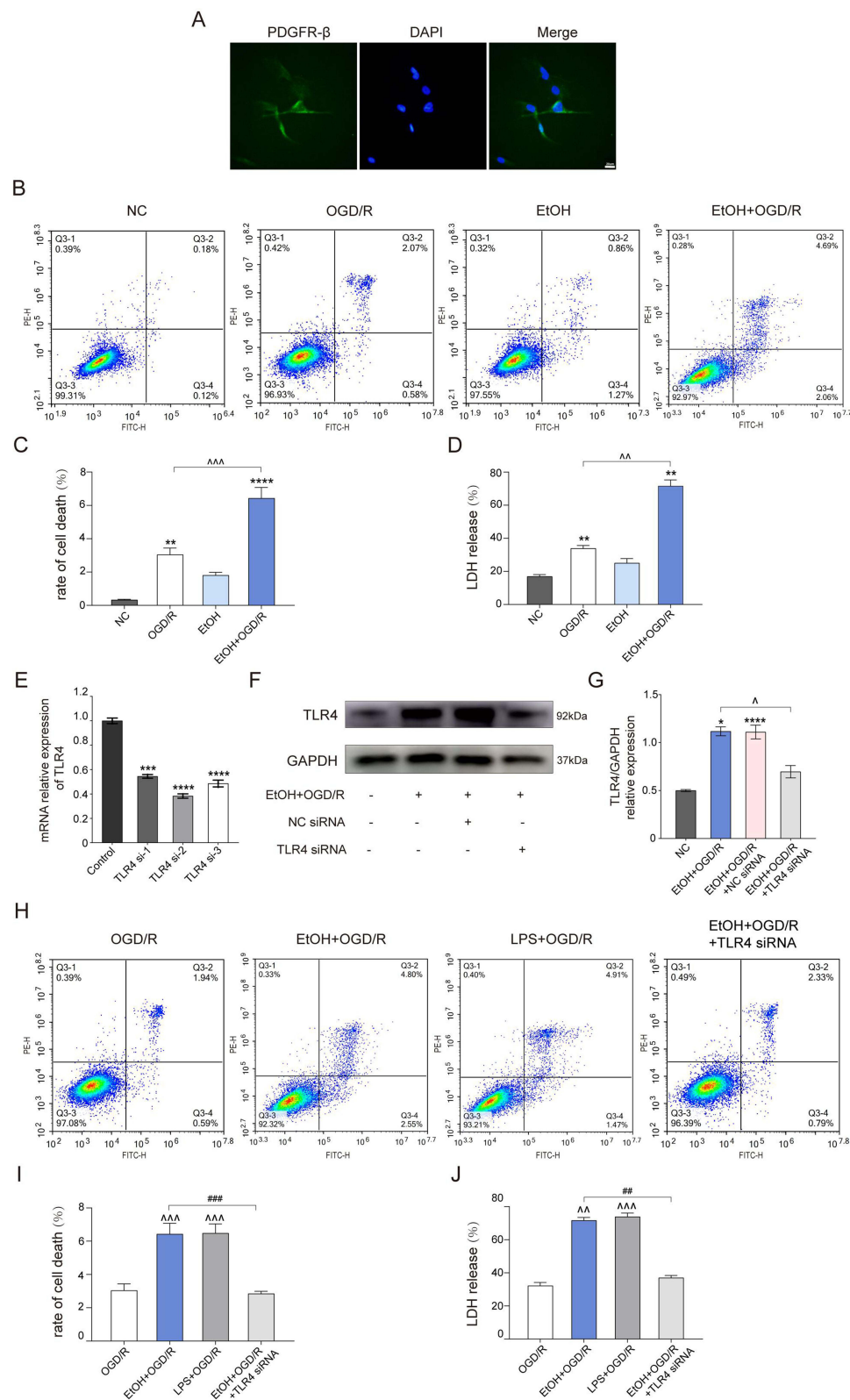
Figure 5 Alcohol increases pericyte mortality and leakage after OGD/R. (**A**) The purity of primary pericyte. Scale bar = 20 μm. (**B** and **C**) Flow cytometry detection of the rate of pericyte mortality in the different groups (n = 3). (**D**) The release of LDH was measured among the groups (n = 3). (**E**) The downregulation efficiency of siRNAs, as measured by RT-qPCR (n = 3). (**F** and **G**) WB detection of the expression levels of TLR4 protein in different groups (n = 3). (**H** and **I**) Flow cytometry detection of the rate of pericyte mortality in the different groups (n = 3). (**J**) The release of LDH was measured among the groups (n = 3). *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001 versus control, ^p < 0.05, ^^ p < 0.01, ^^^p<0.001 versus OGD/R, #p < 0.05, ##p < 0.01, ###p<0.001 versus EtOH+OGD/R.

Table 2 Target Sequences of Three siRNAs

siRNA	Target Sequence
TLR4 siRNA-1	GCTATAGCTTCACCAATTT
TLR4 siRNA-2	GCAGCAGGTCGAATTGTAT
TLR4 siRNA-3	CCTAGAACATGTGGATCTT

Supernatants were collected from pericyte cultures and the levels of cytokines were detected by ELISA assay. The secretion of IL-18 and IL-1β was found to be significantly increased by EtOH (P=0.0065 and 0.0007, respectively) or LPS (P=0.0112 and 0.0003, respectively) treatment following OGD/R. TLR4 blocking alleviated the increment in cytokines concentration. The EtOH+OGD/R+TLR4 siRNA group demonstrated a reduction in IL-18 (P=0.018) and IL-1β (P=0.0008) in comparison to the EtOH+OGD/R group (Figure 7D). This further suggests the importance of pericyte pyroptosis and the inflammatory effect in the pathological process of alcohol-affected stroke.

Inhibition of TLR4 Alleviated the Exacerbating Effect of Alcohol on I/R Injury in Rats

To further validate in animal experiments, rats were intraperitoneal injected with LPS, and significantly increased cerebral infarction area of brains was observed after tMCAO (Figure 8A and B) (P=0.0071), with a corresponding degree of motor decline in behaviour experiments (all P < 0.001) (Figure 8C-E) and cerebral edema increase (P=0.0026) (Figure 8F). Moreover, after intraperitoneal injection of TAK-242 in rats, the amplifying effect of alcohol on cerebral infarction was alleviated (P=0.005) (Figure 8A and B). After I/R injury, the neurological scores (P=0.0141) and movement functions (all P < 0.01) of the EtOH+tMCAO+TAK-242 group improved compared with those of the EtOH+tMCAO group (Figure 8C-E), and ischemic cerebral edema was ameliorated (P=0.0014) (Figure 8F). WB results also demonstrated that LPS argued the MMP-9 protein increase, causing Occludin decrease in rats' brains, compared with the tMCAO group. The TAK-242+EtOH+tMCAO group showed a reduction of MMP-9 (P=0.002), and an increase of the Occludin (P=0.0126), compared with the EtOH+tMCAO group (Figure 2D and E). Blockade of TLR4 attenuates alcohol-induced ischaemic brain injury.

Discussion

In this study, we investigated the effects of long-term alcohol consumption on rats with IS, and the results showed it aggravated I/R injury, as revealed by increased infarct size and functional deficits in rats following stroke. Similar to our results, studies have reported large ischemic lesions caused by alcohol-induced neurovascular inflammation in mice with alcohol exposure following IS.[26] These results are consistent with most current clinical and epidemiological studies showing that stroke risk is linearly associated with heavy alcohol consumption. However, the incidence and prognostic effects of alcohol on IS remain debatable.[27–29] Studies have reported that ethanol consumption is a low-risk factor for adverse cardiovascular events,[28,29] and that the soundness impacts of alcohol consumption is probably due to a specific intake mode. The light-to-moderate alcohol consumption seems to be related to lessening the incidence of myocardial infarction. However, the correlation between alcohol intake and stroke appears to vary according to consumption method because the adverse effect of alcohol on stroke is long-term, requiring cumulative exposure.

In 1983, researchers found that the mortality rate after cerebral infarction increased in chronically drinking gerbils. Subsequently, several studies showed that heavy ethanol consumption exacerbates post-ischemic brain damage, the mechanisms mainly including microglia activation and neutrophil infiltration. Ethanol appears to increase pro-inflammatory and cytokines/chemokines in a dose-dependent manner.[30] Several studies have reported that long-term alcohol-induced neurovascular injury is associated with BBB dysfunction. Rubio-Araiz et al reported significant reductions in the basement membrane and tight junction proteins in postmortem alcoholic human brains.[19] BBB is a highly selective semipermeable membrane formed primarily by microvascular ECs. Pericytes and astrocytes are structurally tightly bound to ECs, ensuring the compactness of the BBB structure and maintaining normal brain homeostasis.[31] In our study, alcohol consumption enhanced EC apoptosis in the ischemic penumbra followed by IS. Additionally, we observed
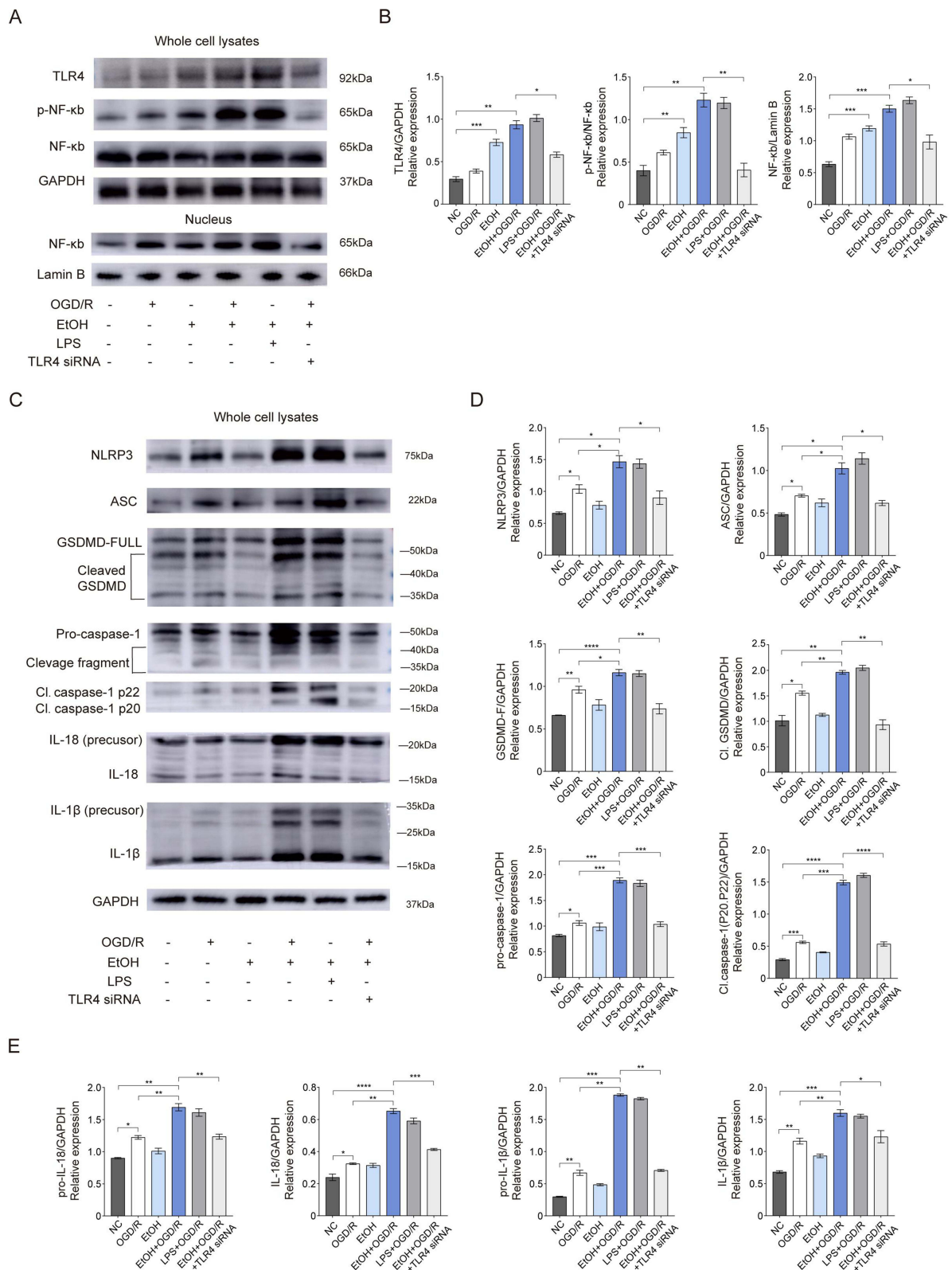
Figure 6 Alcohol increase the activation of pericyte NLRP3 inflammasome in OGD/R via activating TLR4/NF-κB pathway. (**A** and **B**) Representative immunoblot and the normalized protein levels of TLR4, NF-κB, phospho-NF-κB (p-NF-κB) in whole cell lysates and nucleus of pericyte (n = 3). (**C-E**) Representative immunoblot and the normalized protein levels of NLRP3, ASC, full and cleaved GSDMD, pro and cleaved caspase-1, pro-IL-18, IL-18, pro-IL-1β and IL-1β in whole cell lysates of different treated pericytes (n = 3). *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001.
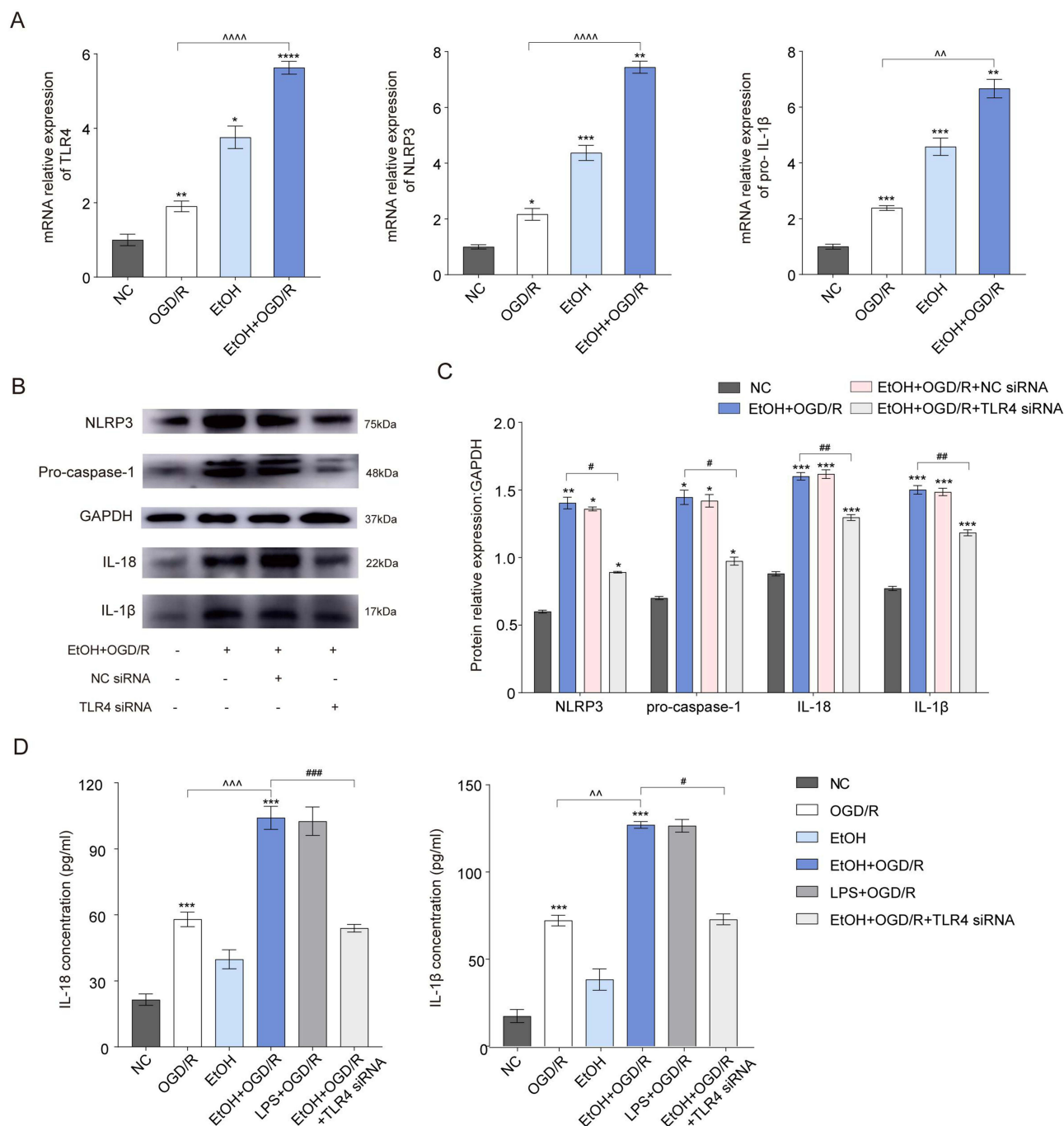
Figure 7 Alcohol pre-treatment elevates NLRP3 and pro-IL-1β mRNA levels. (**A**) Statistic bar graph of relative mRNA levels of TLR4, NLRP3 and pro-IL-1β (n = 3). (**B** and **C**) Representative immunoblot and statistical comparison of NLRP3, pro-caspase-1, IL-18 and IL-1β in whole cell lysates of different treated pericyte (n = 3). (**D**) The IL-18 and IL-1β content in pericyte culture supernatants was determined by ELISA (n = 3). *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001 versus control, ^p < 0.05, ^^ p < 0.01, ^^^p<0.001 versus OGD/R, #p < 0.05, ##p < 0.01 versus EtOH+OGD/R.

increased and decreased expression of MMP-9 and endothelial tight junction proteins compared to non-drinking tMCAO rats' brains, respectively, indicating a worse disruption BBB in the alcohol intake group after tMCAO.

Neuroinflammatory response is a primary cause of BBB dysfunction. As a crucial neurovascular structure, pericytes play a vital role in regulating the BBB function by affecting around cells, for example, changing typical BBB gene molecules in ECs and end-feet polarization of astrocytes.[32] In IS, structural and functional alterations in pericytes and ECs are the main mechanisms of early BBB leakage. In the tMCAO model, pericyte-deficient mice have increased
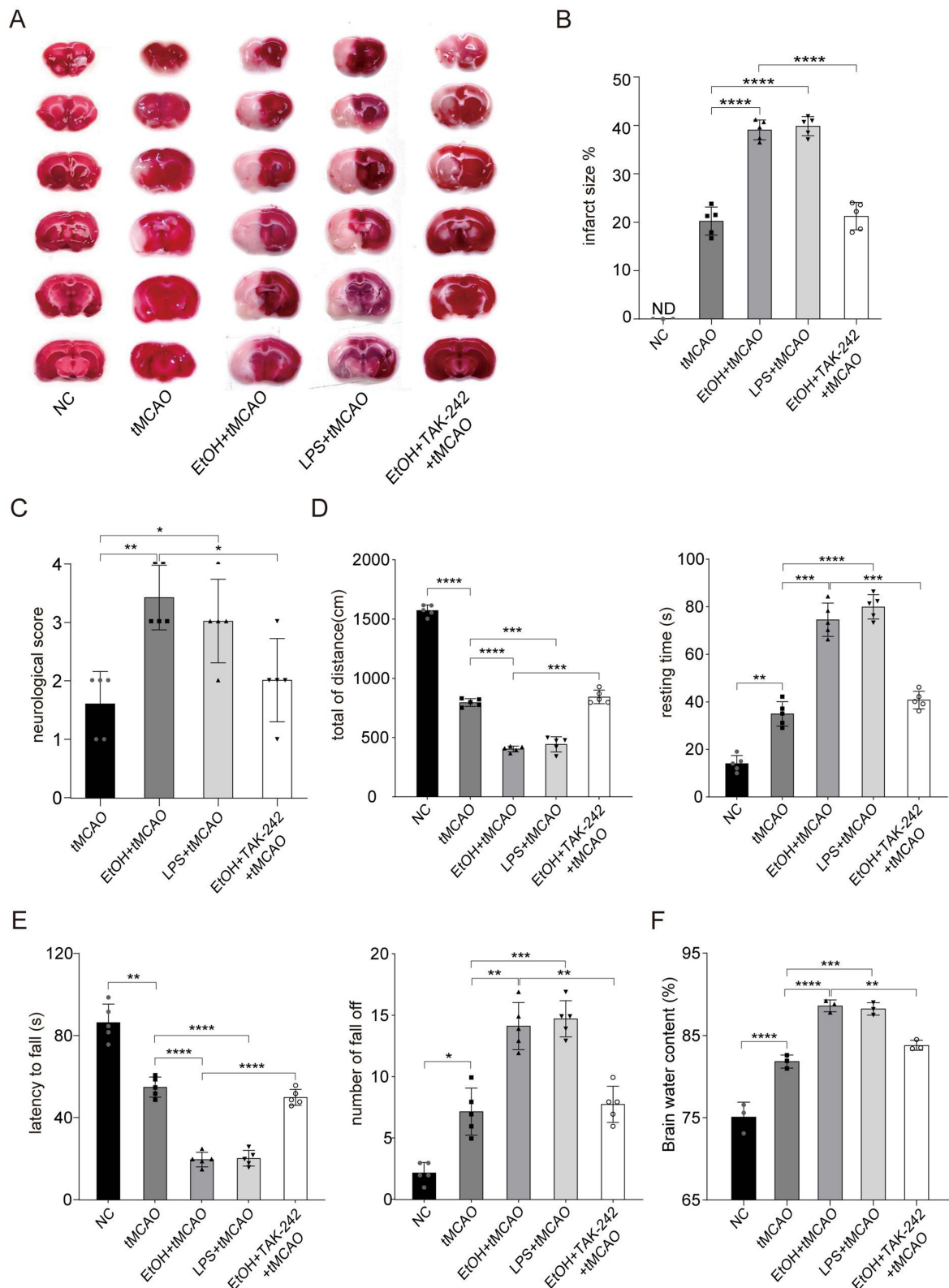
Figure 8 Inhibition of TLR4 attenuates the exacerbating effect of alcohol on I/R injury in rats. (**A**) TTC staining was used to detect infarct size. (**B**) The quantitative analysis of average infarct size (n = 3). (**C**) Neurological scores of each group (n = 5). (**D**) Statistic bar graph of the total moving distance (cm) in 2 min and the resting time (s) of each group in the open field test (n = 5). (**E**) Statistic bar graph of the holding time on the rotating bar (s) and the total fall-off numbers of each group in the rotarod test (n = 5). (**F**) Average brain water contents in different groups (n = 3). ND means "not detected", *p < 0.05, ** p < 0.01, ***p<0.001, ****p<0.0001.

cerebral infarct size and cerebral edema compared to wild mice.[33] In addition, the contractile properties of pericytes can cause segmental narrowing of capillaries and reduced blood flow after IS, which are associated with the "no-reflow" of the microcirculation after I/R. Importantly, pericytes have many of the properties of immunomodulatory cells and participate in the immune response by upregulating cell adhesion molecules, secreting inflammatory factors, and presenting antigens. Recent studies have emphasized that pericytes play a neuroinflammatory role when stimulated, thus promoting BBB destruction.[34]

NLRP3 inflammasome is the crucial player in pyroptosis and is the most studied inflammasome associated with central nervous system-related diseases. It is contributing to the pathological process of ischemic brain injury.[35] Inhibiting NLRP3 inflammasome activation is a proven prevention and treatment strategy for IS.[5,36] Compared to the sham surgery group, we found a higher expression of NLRP3 and IL-1β in the pericytes at the ischemic penumbra in the tMCAO group. Consistent with a vitro study, an immortalized cell line of human brain vascular pericytes showed increased expression of pyroptosis-associated proteins after OGD/R treatment.[37] To further explore the mechanisms underlying alcohol-mediated aggravation of stroke injury, we examined neuroinflammation in alcohol-fed rats after tMCAO. We surprisingly found that the NLRP3 inflammation was overactive in the EtOH+tMCAO group compared with tMCAO only. However, we did not find significant NLRP3 inflammasome activation in the EtOH groups. Noticeably, the TLR4 was activated by alcohol intake in rats' brains. Thus, this suggests that alcohol intake directly activates the expression of TLR4, which is known to cross-talk with the NLRP3 signaling pathway, and hence likely has a contributory role in the activation of peridermal NLRP3 inflammasomes in I/R injury.

To further explore the machine that alcohol acts on pericytes to affect their inflammatory response. In vitro experiments were conducted, and the results revealed that alcohol treatment resulted in increased pericyte death after OGD/R by flow cytometry, accompanied by more cell leakage, which could be due to the rupture of cell membranes in cell pyroptosis. As is known that the TLR4/NF-κB signaling pathway can affect the secretion of cytokines and chemokines[38] thus impacting cell apoptosis. We used LPS, the specific TLR4 stimulator,[39] to treat pericytes instead of alcohol before OGD/R, and observed a similar increase in pericyte mortality rate and LDH release with the EtOH+OGD/R group. And we used TLR4siRNA to knock down the target gene in the pericytes. It lessened the exaggerated death of pericytes and over-release of LDH caused by alcohol. These results indicate that alcohol activates TLR4 in pericytes, aggravating cell death and neuroinflammation.

Numerous experimental results revealed that alcohol induces the activation of transcription factors, such as NF-κB and interferon regulatory factor 3,[40] by stimulating TLRs and enhancing the downstream pathways, which can induce the transcription of inflammatory genes, causing neuroinflammation. Among these, TLR4 is considered a major factor for alcohol-induced neuroimmune activation. Activation of the TLR4/NF-κB pathway is a fundamental step in NLRP3 inflammasome formation.[22,36] Reactive oxygen species overproduction, lysosomal rupture, and K^+ efflux are the three main NLRP3 inflammasome activation pathways.[41]

TLR4-mediated NLRP3-inflammasome activation was observed in microglia cells,[20] and alcohol treatment of astrocytes induced the release of inflammatory factor-rich extracellular vesicles by activating the TLR4/NF-κB pathway,[21] thus contributing to ethanol-induced neuroinflammation. However, pericytes are involved in the amplification of the alcohol-induced neuroinflammatory cascade through TLR4 activation. As far as we know, this is the first study to report elevated TLR4 expression in primary rat brain pericytes treated with sterile alcohol.

Consistent with animal studies, we found that TLR4 and its downstream target p-NF-κB were active in the alcohol treated group, while the NF-κB translocation to nuclear was increased, indicating the activation of the TLR4/NF-κB pathway. Also, the levels of NLRP3 and its downstream proteins were significantly increased by alcohol pretreatment during OGD/R and were significantly higher than those observed without alcohol. An increase in cytokine IL-18 and IL-1β secretion due to pericyte pyroptosis was also observed, implying that pericyte can affect the death of its adjacent ECs by cytokines produced.

We wonder how the activation of TLR4 by alcohol affects the activation of NLRP3 inflammation after OGD/R, and conduct a series of RT-qPCRs to evaluate the mRNA levels of TLR4/NLRP3-associated genes. Our results suggest that alcohol treatment upregulates the transcript levels of NLRP3 and pro-IL-1β mRNA. Although it did not directly increase NLRP3 inflammasome proteins in the pericytes, it was associated with exacerbated inflammatory responses when

pericytes received a secondary insult, in this case, hypoxia. LPS has been found to activate TLR4 in pericytes further upregulate NLRP3 mRNA and pro-IL-1β transcripts.[39] High levels of NLRP3 and pro-IL-1β mRNA are critical for the formation of an effective inflammasome, so upon encountering I/R injury, increased mRNA expression provides the basis for the subsequent inflammation overactivation (Figure 9). Contrary to our study, another study stimulated pericytes with H_2O_2 to simulate oxidative stress in I/R insult. The NLRP3 inflammasome was upregulated in the brain pericytes; however, no significant changes were observed in TLR4 expression.[17] In contrast, an elevation in TLR4 expression was found in pericytes in the tMCAO model, according to our results. The discrepancy is most probably due to the different treatment methods used.

To further probe whether alcohol pretreatment increases NLRP3 inflammasome activation after OGD/R in a TLR4-dependent manner, we used TLR4 siRNA and found excessive activation of inflammasomes by alcohol is largely abolished. LPS pretreatment magnified the activation of inflammasomes by OGD/R injury, which was similar to the activation induced by alcohol pretreatment. In the in vivo experiment, more severe infarcts and neurological deficits were shown in the LPS+tMCAO group than those in the tMCAO group. We selected TAK-242, a selective inhibitor of TLR4, for intraperitoneal injection in rats in the alcohol intake group and found that the behavioral performance of the rats was improved than the vehicle group after prolonged tMCAO. Although the cerebral infarction size and neurological scores were improved after TLR4 receptor blockade in rats, they were not totally consistent with those in the tMCAO group, suggesting that alcohol may also act adversely through other potentially complex mechanisms in stroke. In contrast, other studies have shown that LPS induces neuroprotection by pre-activating TLR4. Hayakawa et al found that LPS preconditioning attenuates cell apoptosis, protects against cytotoxic effects after spinal cord injury, and reduces post-injury apoptosis.[42] In these studies, researchers added LPS or other stimuli as an analogous but faint noxious stimulus near the damage site,[43] such that the procedure induces tolerance and protection against subsequent damage. This discrepancy
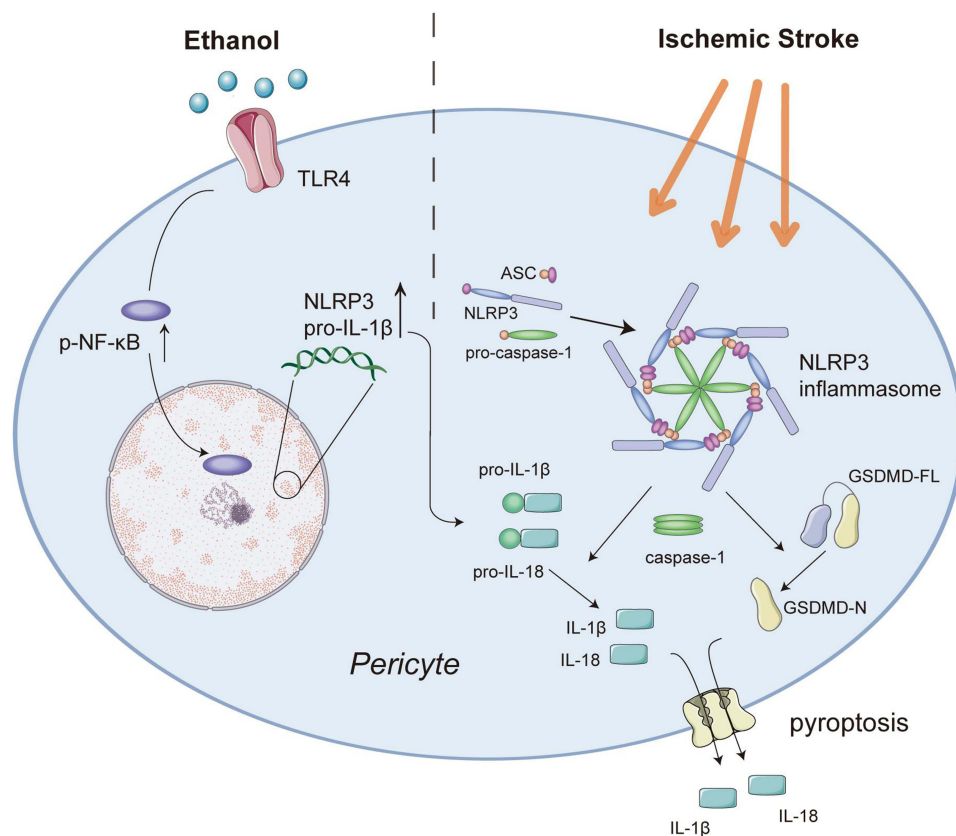
Figure 9 Schematic illustration describing the underlying mechanism of alcohol pre-activated TLR4, which increased pericyte pyroptosis after I/R injury. Alcohol binds to TLR4 in the pericytes and activates its downstream p-NF-κB, which further upregulates the transcript levels of NLRP3 and pro-IL-1β mRNA. Upon encountering factors caused by I/R injury, NLRP3 inflammasomes are over-activated. This figure was drawn with Adobe Illustrator (USA).

might be due to the difference in types or times of the pre-conditioned stimuli. The stimuli that produce protective effects are often weak and time-limited.

However, this study is not without limitations. First, constrained by practicalities, a larger sample size is required to infer more reliable results. In addition, we used TLR4 inhibitor intraperitoneal injection, which is widely used in previous research, but it would be more convincing to knock out the rat TLR4 gene specifically. Lastly, the mechanisms of how pericyte pyroptosis-mediated neuroinflammation triggered by alcohol exacerbated post-stroke brain injury deserve further studies, including BBB destruction, death of other surrounding cells, etc.

In summary, our study advances the notion that long-term alcohol intake contributed to cerebral injury caused by IS in rats. A possible mechanism underlying alcohol accelerating neurological deficit may involve NLRP3 inflammations activation, via activating TLR4/NF-κB pathway in pericyte. Inhibition of TLR4 in the pericytes alleviated the excessive neuroinflammation during I/R injury induced by alcohol. These preliminary results provide not only an adequate understanding of the alcohol's role and pericyte pyroptosis in I/R injury but also offer insights into potential applications for the prevention and therapeutic targets for ischemic stroke.

Conclusion

Long-term alcohol consumption pre-activates TLR4 in pericytes, and based on the activation of the NLRP3 inflamma-some in pericytes induced by ischemic brain injury, the pre-activated TLR4/NF-κB pathway further engages the NLRP3 inflammasome activation, inducing neuroinflammatory cascade responses and exacerbating I/R injury.

Data Sharing Statement

The original contributions presented in the study are included in the article/Supplementary Material. Any further materials required can be provided by the corresponding author (gongzhe1415@163.com).

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest.

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