Submitted: 22 July, 2025 Accepted: 22 August, 2025 Published: 08 October, 2025 DOI:10.22514/sv.2025.148

Open Access
Signa Vitae

ORIGINAL RESEARCH

Tormentic acid attenuates neuronal injury caused by ischemia-reperfusion via suppression of microglial mediated neuroinflammation

Can Chen[1,*,†], Yue Dong[2,†]

[1]Department of Neurology, Sir Run Run Hospital Nanjing Medical University, 211000 Nanjing, Jiangsu, China
[2]Department of Pancreatic Surgery, Sir Run Run Hospital Nanjing Medical University, 211000 Nanjing, Jiangsu, China

***Correspondence**
chencanchencan1122@163.com
(Can Chen)

[†] These authors contributed equally.

Abstract

Background: To explore protective impact and underlying processes of tormentic acid (TA) against microglial-mediated neuroinflammation and oxygen-glucose deprivation/reoxygenation (OGD/R)-induced neuronal injury. **Methods**: HT22 neuronal cells and BV2 microglial cells underwent OGD/R to mimic ischemia-reperfusion injury *in vitro*. TA was administered at various concentrations before the OGD/R procedure. Cells viabilities were evaluated using cell counting kit-8 (CCK8) and Lactate Dehydrogenase (LDH) assays, while Western blotting and flow cytometry (FCM) were employed to assess apoptosis. Inflammatory cytokines were quantified by quantitative Polymerase Chain Reaction (qPCR) and Enzyme-Linked Immunosorbent Assay (ELISA). The involvement of the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) axis was examined with Western blotting. **Results**: TA significantly enhanced cell viability in HT22 cells stimulated by OGD/R and reduced LDH release. TA markedly inhibited neuronal apoptosis induced by OGD/R. Furthermore, TA suppressed pro-inflammatory cytokines' production for BV2 microglia after OGD/R exposure. Conditioned media from TA-treated microglia attenuated apoptosis in co-cultured HT22 neurons. Furthermore, TA inhibited NF-κB activation in both microglial and neuronal cells. **Conclusions**: Tormentic acid exerts neuroprotective effects regarding ischemia-reperfusion injury by reducing microglial-mediated neuroinflammation and suppressing NF-κB axis.

Keywords

Tormentic acid; Ischemia-reperfusion; Neuroinflammation; Microglia; NF-κB pathway

1. Introduction

Ischemic stroke is among the major reasons of mortalities globally. It is symbolized by the abrupt cerebral blood flow interruption [1]. Acute ischemic stroke (AIS) often results in irreversible neuronal damage and long-term neurological deficits, posing substantial impact on healthcare systems and public health [2]. In particular, a pathological process of cerebral ischemia-reperfusion (I/R) injury happens following the blood supply restoration towards ischemic brain region, frequently exacerbates neuronal death and neurological dysfunction [3]. This reperfusion-associated injury triggers a cascade of detrimental events, including excitotoxicity, mitochondrial dysfunction, and intense neuroinflammatory responses [4]. Among these, neuroinflammation mediated by microglia has a central function of amplifying neuronal injury. Microglia being central nervous system's resident immune cells are speedily triggered in response to ischemic insults [5]. These cells release inflammatory mediators that not only exacerbate neuronal injury but also interfere with neuronal survival signaling pathways. The NF-κB is among pivotal regulator of microglial activation and neuroinflammatory responses during ischemic stroke [6]. NF-κB activation contributes to numerous pro-inflammatory genes' transcription and promotes neuronal apoptosis [7]. NF-κB pathway inhibition attenuates the ischemic injury and improves neurological results in experimental stroke model.

Tormentic acid (TA) being a natural triterpenoid compound enriched from the bark of medicinally important plants, exhibits potent antioxidant, anti-inflammatory, and neuroprotective characteristics [8]. Earlier research have shown that TA can inhibit Interleukin-1 beta (IL-1β)-induced chondrocyte apoptosis, alleviate hepatic fibrosis and inflammation, and protect endothelial cells from hypoxia-induced apoptosis [9]. Moreover, TA exerts protective effects in neurodegenerative disease models by modulating Wnt/β-catenin and other survival-related pathways [10]. Notably, TA's pentacyclic triterpenoid structure confers unique pharmacokinetic advantages, including blood-brain barrier permeability and multi-target modulation, positioning it as a superior candidate over conventional single-target inhibitors [8–10]. However, the

potential role and underlying mechanisms of TA in microglial-mediated neuronal injury and neuroinflammation in cerebral I/R injury remain poorly understood.

Although TA's neuroprotective properties have been reported in neurodegenerative models, its role in microglial-mediated neuroinflammation during cerebral I/R injury is not completely understood. Thus, this study aimed to investigate neuroprotective impact of tormentic acid against microglial-induced neuroinflammation and I/R neuronal injury via OGD/R model *in vitro*. Unlike prior studies focusing solely on neuronal or peripheral anti-inflammatory effects, our work uniquely investigates TA's capacity to disrupt the vicious cycle of microglial activation and neuronal apoptosis via NF-κB inhibition, thereby addressing a critical gap in stroke therapeutics. This dual cell targeting strategy underscores TA's potential as multimodal therapeutical compound for I/R injury.

2. Materials and methods

2.1 Cells culturing and treatment

The American Type Culture Collection (ATCC, Manassas, VA, USA) provided murine microglial BV2 cells and mouse hippocampal neuronal HT22 cells. Cell culturing was done in (DMEM, Gibco, 11965092, Grand Island, NY, USA) having 10% FBS (Gibco, 10099141C, Grand Island, NY, USA) in humid incubator at 37 °C with 5% CO_2. Tormentic acid (TA; Sigma-Aldrich, St. Louis, MO, USA, T2815, Lot# 12345; $\geq$98% purity by HPLC, stored at −20 °C in desiccated aliquots) was dissolved in endotoxin-free, sterile-filtered Dimethyl Sulfoxide (DMSO, Sigma, St. Louis, MO, USA, D2650, Lot# 67890) to prepare a 10 mM stock, with final DMSO concentration maintained at $\leq$0.1% (v/v), a level confirmed non-toxic in pilot studies. Tumor Necrosis Factor-alpha (TNF-α, Sigma,100 ng/mL, H8916) was used in HT22 cells for 24 h. Cells for OGD/R model had been incubated in glucose-free and serum-free DMEM (Gibco, Grand Island, NY, USA, 11966025) within tri-gas incubator (Thermo Scientific, Waltham, MA, USA, Heracell™ VIOS 160i) in hypoxic environment (94.5% N_2, 5% CO_2, and 0.5% O_2) for 12 h at 37 °C, followed by reoxygenation in complete medium for 24 h, as previously described [10]. For transfection assays, siRNA or plasmids underwent transfection by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA, L3000008).

2.2 Cells viability and LDH release analyses

Cells viabilities were evaluated by CCK8 (Beyotime, Shanghai, China, C0037) assay. CCK8 reagent (10% v/v in culture medium) was employed to incubate cells in dark at 37 °C for 2 h. Microplate reader (BioTek, Synergy H1, Synergy H1, Winooski, VT, USA) was used to record absorbance at 450 nm, with 650 nm as reference wavelength. Preliminary validation confirmed a linear detection range (0.2–2.0 OD units) and excluded edge effects by utilizing central wells of the plate. LDH Cytotoxicity Assay Kit (Beyotime, C0016, Shanghai, China) was employed to measure LDH release at 490 nm. Supernatants from cultures were gathered after 24 h of reoxygenation and centrifuged for 5 min at 300 × g. Reaction mix (catalyst:dye = 1:45) was added to samples, followed by incubation at 25 °C for 30 min protected from light. The wavelength of 490 nm was set to record absorbance (background subtraction at 680 nm). Cells were lysed by 1% Triton X-100 to determine total LDH release (100% control).

2.3 Flow cytometric analysis for apoptosis

Annexin V-Fluorescein Isothiocyanate (V-FITC)/Propidium Iodide (PI) Apoptosis Detection Kit (Beyotime, C1062M, Shanghai, China) as per the company guidelines was applied to detect apoptotic cells with following specifications: Phosphate-Buffered Saline (PBS) was used to wash cells (1×10^6/sample), followed by staining at 25 °C for 15 min in dark with 10 μL PI (20 μg/mL) plus 5 μL Annexin V-FITC. Analysis was performed on a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) using the following gating strategy: (1) Forward Scatter-Area/Side Scatter-Area (FSC-A/SSC-A) plots excluded debris and doublets; (2) Fluorescence compensation was adjusted using single-stained controls; (3) Quadrant gates were set to distinguish late apoptotic/necrotic (Annexin V^+/PI^+), early apoptotic (Annexin V^+/PI^-), and viable (Annexin V^-/PI^-) populations. Minimum 10,000 events were attained for each sample. FlowJo v10.8 (BD Biosciences, San Jose, CA, USA) was employed to analyze the data with apoptosis rate calculated as percentage of Annexin V^+ cells (Q2 + Q3).

2.4 ELISA assays

TNF-α, IL-6, and IL-1β concentrations from culture supernatants had been quantified using mouse ELISA kits (Beyotime, Shanghai, China, PI301, PI326, and PT512 respectively) following an optimized protocol. Lyophilized standards were reconstituted in assay diluent to generate stock solutions (TNF-α: 1000 pg/mL, IL-6: 500; IL-1β: 1000), followed by preparation of 7-point serial dilutions in culture medium for standard curves. Samples and standards (100 μL/well) were assayed in duplicate with incubation at 37 °C for 2 h. Post washing, biotinylated detection antibody (100 μL) had been added at 37 °C for 1 h, followed by horseradish peroxidase (HRP)-conjugated streptavidin (100 μL, 30 min) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate development (15 min). 2 M Sulfuric acid (50 μL) was added to stop reaction, followed by measuring absorbance on BioTek Synergy H1 microplate reader (BioTek, Winooski, VT, USA) at 450 nm (with 570 nm correction). All assays met quality control criteria (standard curve R^2 >0.99, inter-assay coefficient of variation (CV) <15%). Samples exceeding the standard range were diluted and reanalyzed. Data were analyzed using four-parameter logistic curve fitting in GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

2.5 Quantitative rt-PCR

RNAiso Plus (Takara, 9108, Takara Bio Inc., Kusatsu, Shiga, Japan) was employed to extract total RNA, HiScript II Q RT SuperMix kit (Vazyme, R223-01, Nanjing, Jiangsu, China) to perform reverse transcription, and QuantStudio 5 Real-Time PCR System (Thermo Fisher, Waltham, MA, USA)

via ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711, Nanjing, Jiangsu, China) to conduct qPCR. Primers were: IL-1β-F: 5′-GCAACTGTTCCTGAACTCAACT-3′; IL-1β-R: 5′-ATCTTTTGGGGTCCGTCAACT-3′; IL-6-F: 5′-TAGTCCTTCCTACCCCAATTTCC-3′; IL-6-R: 5′-TTGGTCCTTAGCCACTCCTTC-3′; TNF-α-F: 5′-CCCTCACACTCAGATCATCTTCT-3′; TNF-α-R: 5′-GCTACGACGTGGGCTACAG-3′; β-actin-F: 5′-GGCTGTATTCCCCTCCATCG-3′; β-actin-R: 5′-CCAGTTGGTAACAATGCCATGT-3′.

2.6 Western blot analysis

Fractionation of protein samples (30 μg per lane) was made via 10% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE), followed by their transfer to Polyvinylidene difluoride (PVDF) membrane (Millipore). Blocking was done at room temperature for 1 h by 5% non-fat milk, followed by incubating membranes at 4 °C overnight with primary antibodies (all from Abcam, Cambridge, UK): anti-β-actin (ab8226, 1:5000), anti-IκBα (ab76429, 1:1000), anti-p-IκBα (ab133462, 1:1000), anti-p65 (ab16502, 1:1000), anti-p-p65 (ab76302, 1:1000), anti-TNF-α (ab183218, 1:1000), anti-IL-6 (ab233706, 1:1000), anti-IL-1β (ab283818, 1:1000), anti-Bcl-2 (ab182858, 1:1000), and anti-Bax (ab32503, 1:1000). After Tris-Buffered Saline with Tween 20 (TBST) washes, secondary antibodies conjugated with HRP (Abcam, 1:5000, Cambridge, UK) were employed to incubate membranes at room temperature for 1 h. Enhanced ChemiLuminescence (ECL) reagents (Thermo Fisher, Waltham, MA, USA) were used to visualize protein bands and densitometry (Image Lab 6.1, Bio-Rad Laboratories, Hercules, CA, USA) for quantification. β-actin was employed as the loading control, and all experimentation included molecular weight markers and positive controls.

2.7 Statistical analysis

All quantitative data were presented in the form of mean $\pm$ Standard Deviation (SD) for experiments conducted in triplicate. Shapiro-Wilk test ($p > 0.05$) was applied to confirm normality and Brown-Forsythe test for variance homogeneity. For multiple group comparisons (*e.g.*, dose-response experimentation), Tukey's *post hoc* test plus one-way analysis of variance (ANOVA) was applied for normal distribution of the data, while Kruskal-Wallis with Dunn's tests for non-normal distributions. The *t*-test was employed for pairwise comparisons (Welch's corrections upon unequal variances, as determined by *F*-test). GraphPad Prism 9.0 was engaged to perform analysis (α = 0.05, power = 0.8), with sample sizes determined by power analysis to ensure adequate statistical power.

3. Results

3.1 Tormentic acid promotes cells viabilities in OGD/R subjected HT22 cells

Tormentic acid (TA) molecular structure was shown in Fig. 1A. TA effects on cells viabilities in HT22 cells under OGD/R conditions were first evaluated to assess whether TA protects neurons against I/R injury. Treatments by increased TA concentrations (5–100 nM) under normal conditions revealed no obvious cytotoxicity up to 20 nM, whereas higher concentrations (50, 100 nM) significantly reduced viability (Fig. 1B). Under OGD/R insult, HT22 cells exhibited markedly reduced viability and elevated LDH release, both of which were substantially reversed by TA pretreatment at 10 and 20 nM (Fig. 1C,D). These findings indicate that TA enhances neuronal survival and alleviates plasma membrane damage in OGD/R exposed HT22 cells.

3.2 TA reduces apoptosis induced by OGD/R in HT22 cells

We explored if TA neuroprotective impact is linked to apoptosis suppression. Flow cytometry analysis exhibited substantial enhancement in apoptotic cell population after OGD/R, being drastically attenuated via TA treatments (Fig. 2A). Consistently, Western blot analysis showed that TA upregulated anti-apoptotic Bcl-2 protein levels and downregulated pro-apoptotic Bax compared to OGD/R group (Fig. 2B). These results confirm that TA exerts anti-apoptotic effects in HT22 neurons subjected to ischemic stress.

3.3 TA alleviates microglia-mediated neuronal apoptosis

To examine whether TA can mitigate microglial-derived toxicity to neurons, HT22 cells were incubated with conditioned media (CM) collected from differently treated BV2 cells. CM from OGD/R-stimulated microglia ($CM^{OGD/R}$) significantly decreased HT22 viability and induced apoptosis, whereas CM from TA-pretreated OGD/R microglia ($CM^{OGD/R}$ + TA) restored cell viability and reduced apoptotic rates (Fig. 3A,B). Western blotting further confirmed increased Bcl-2 expressions and reduced Bax in the $CM^{OGD/R}$ + TA group (Fig. 3C). Together, these findings indicate that TA protects neurons from secondary injury mediated by activated microglia under ischemic conditions.

3.4 TA inhibits inflammatory cytokine production in OGD/R-stimulated microglia

To investigate whether TA regulates microglial inflammatory responses, we measured protein and mRNA levels of key cytokines for OGD/R exposed BV2 cells. qPCR results showed significant upregulation of transcripts of these factors after OGD/R, which were markedly decreased following TA pretreatment (Fig. 4A). In parallel, both Immunoblot and ELISA assays confirmed a reduction in secreted cytokines in the culture supernatant after TA intervention (Fig. 4B,C). These data demonstrate that TA effectively suppresses OGD/R-induced microglial inflammation at both the transcriptional and protein levels.

3.5 TA inhibits NF-κB signaling activation in both microglia and neurons

Given the NF-κB pivotal function in inflammation and apoptosis, we evaluated whether TA modulates this pathway. In BV2

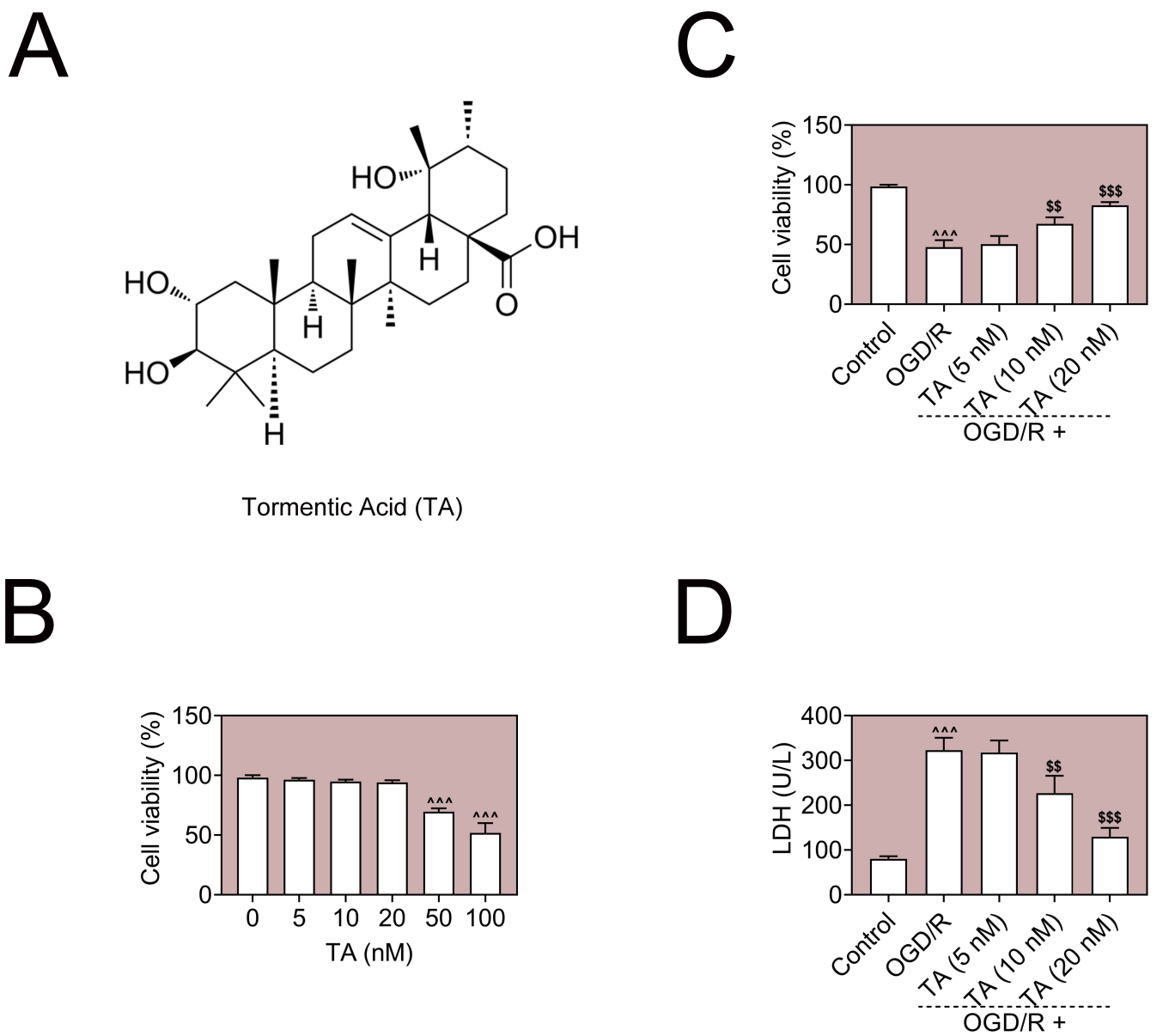

FIGURE 1. Tormentic acid enhances cell viability in HT22 cells under OGD/R conditions. (A) Chemical structure of tormentic acid (TA). (B) HT22 cells were treated with various concentrations of TA (5, 10, 20, 50, 100 nM), and cell viability was assessed by CCK8 assay. (C) HT22 cells were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) with or without TA pretreatment (5, 10, 20 nM), and cell viability was measured. (D) LDH release was evaluated to assess cell injury following OGD/R and TA treatment. Data are presented as mean ± SD (n = 3). Each experiment has been repeated for 3 times. $^{\wedge\wedge\wedge}p < 0.001$ *vs.* Control; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs.* OGD/R group. LDH: lactate dehydrogenase.

cells, OGD/R stimulation significantly increased phosphorylation of p65 and IκBα and decreased total IκBα levels, whereas TA pretreatment markedly reversed these changes (Fig. 5A). Similarly, in HT22 cells cultured with $CM^{OGD/R}$, NF-κB activation was induced, while $CM^{OGD/R}$ + TA mitigated these effects by reducing p-IκBα and p-p65 expression and restoring IκBα level (Fig. 5B). Interestingly, the inhibitor of NF-κB pathway, TNF-α, was further treated into the HT22 cells cultured with $CM^{OGD/R}$ and TA. We noticed the reversal in promotion of cells viabilities, and suppression of cell apoptosis in $CM^{OGD/R}$ cells caused by TA treatment, confirmed by CCK-8, LDH, and FCM assays (Fig. 5C–F). These outcomes demonstrate that TA depicts anti-apoptotic as well as anti-inflammatory impacts by inhibiting the NF-κB cascade in microglia and neurons under ischemic conditions.

4. Discussion

Although advances in thrombolytic and endovascular therapies have improved outcomes for some stroke patients, many still suffer from irreversible neurological damage due to secondary injury triggered by reperfusion. The pathophysiological mechanisms underlying I/R injury are highly complex, involving apoptosis, inflammation, mitochondrial dysfunction, oxidative stress, and excitotoxicity [4, 11]. Out of all processes, neuroinflammation has evolved to have key contribution in neuronal death, particularly during the reperfusion phase. The identification of novel neuroprotective agents is thus required

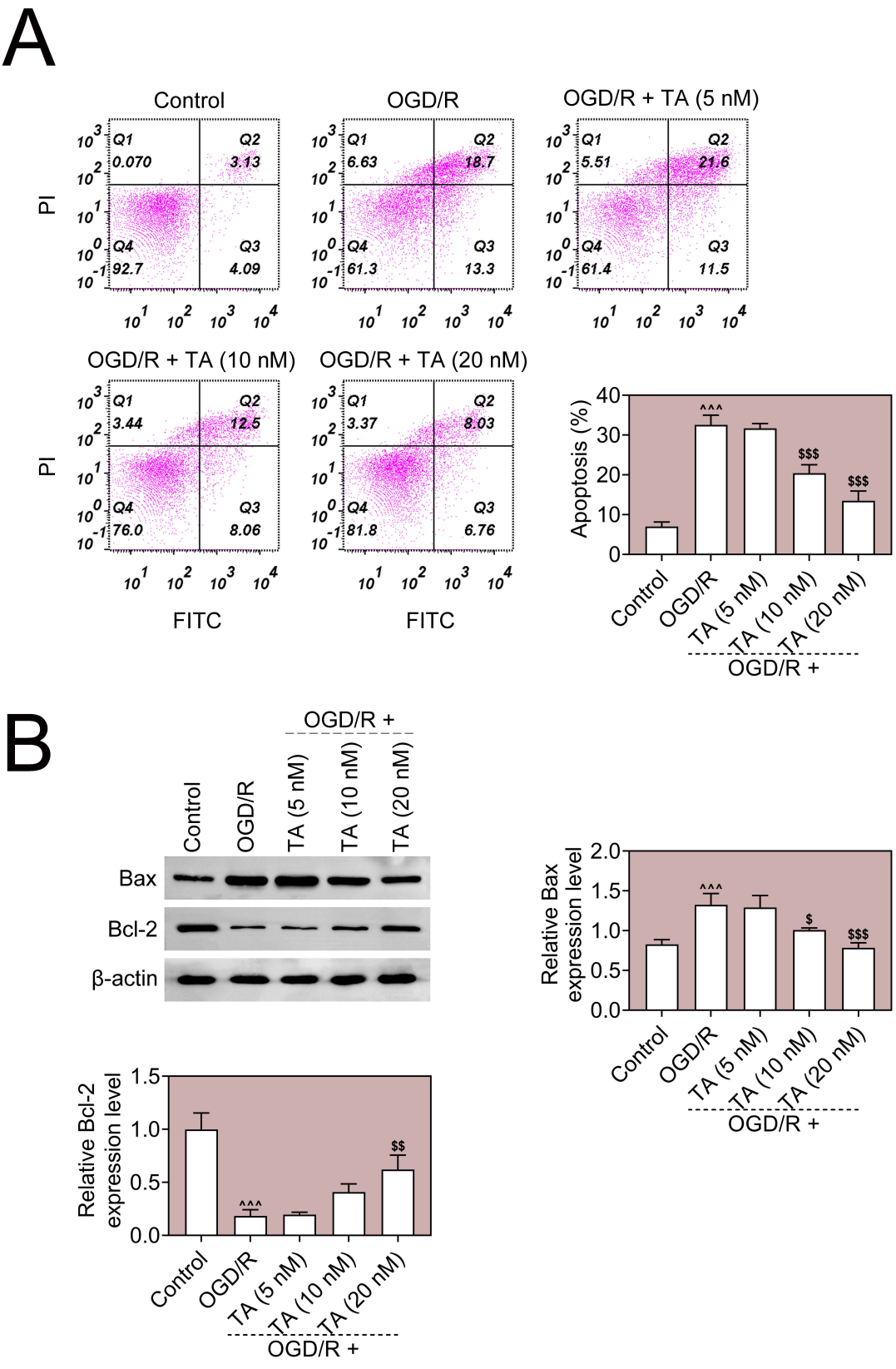

FIGURE 2. Tormentic acid attenuates OGD/R-induced apoptosis in HT22 neuronal cells. (A) Apoptosis of HT22 cells after OGD/R with or without TA pretreatment (5, 10, 20 nM) was detected by flow cytometry using Annexin V-FITC/PI staining. Representative dot plots and quantification of apoptotic cell percentages are shown. (B) Western blot analysis of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in each treatment group. Densitometric quantification of Bax and Bcl-2 relative to β-actin is shown below. Data are presented as mean ± SD (n = 3). Each experiment was independently repeated three times. $^{\wedge\wedge\wedge}p < 0.001$ *vs.* Control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs.* OGD/R group. OGD/R: oxygen-glucose deprivation/reoxygenation; TA: tormentic acid; PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate.

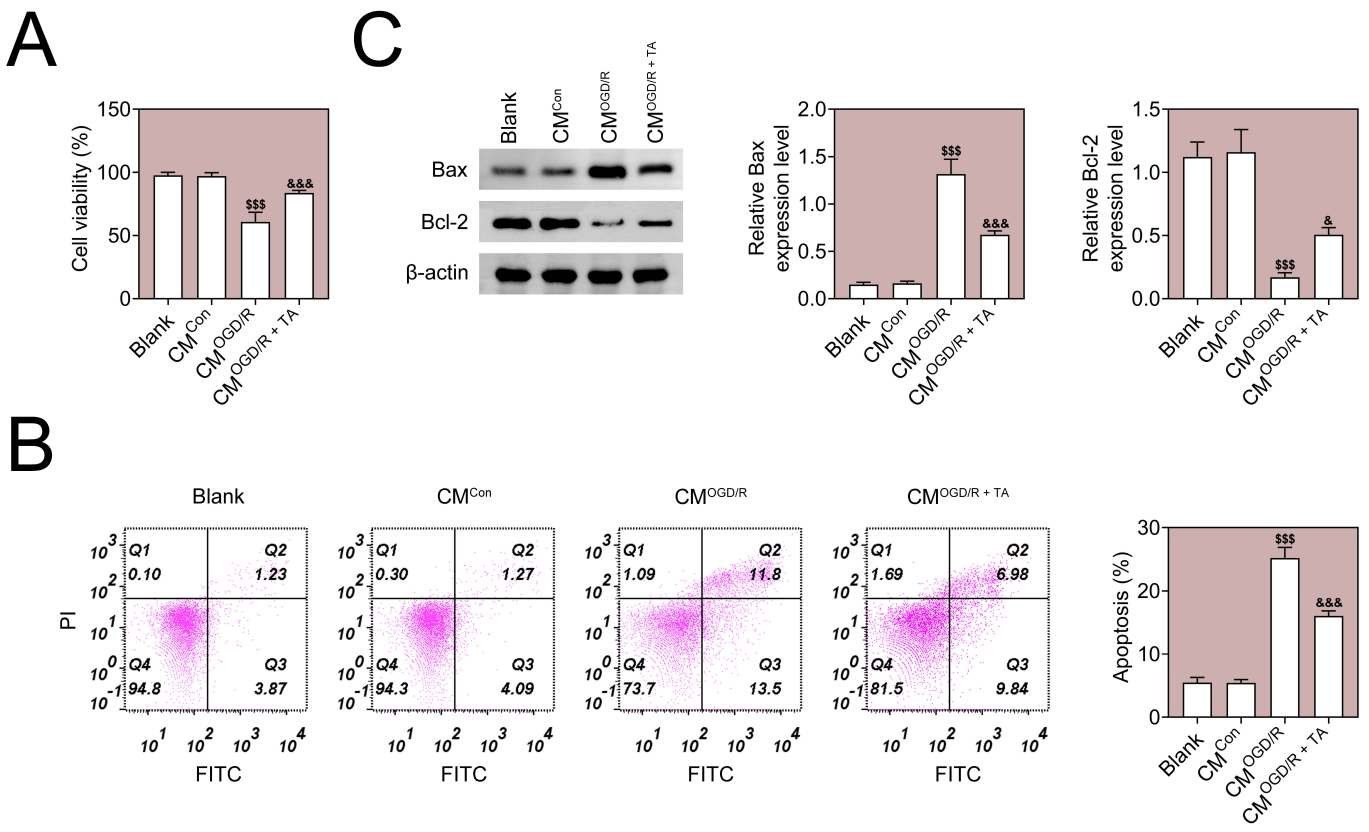

FIGURE 3. Tormentic acid reduces microglia-induced apoptosis in HT22 neurons. (A) HT22 cells were cultured with conditioned media (CM) from control BV2 cells, OGD/R-stimulated BV2 cells, or TA-pretreated OGD/R BV2 cells. Cell viability was assessed using the CCK8 assay. (B) Apoptosis of HT22 cells under different CM treatments was measured by Annexin V-FITC/PI staining and analyzed by flow cytometry. Representative dot plots and quantitative analysis of apoptotic rates are shown. (C) Western blot analysis of Bax and Bcl-2 protein expression in HT22 cells after CM treatments. Quantification of relative protein levels is shown. Data are presented as mean ± SD (n = 3). Each experiment was independently repeated three times. $^{\$\$\$}p < 0.001$ *vs.* Control group, $^{\&\&\&}p < 0.001$, $^{\&}p < 0.05$ *vs.* $CM^{OGD/R}$ group. OGD/R: oxygen-glucose deprivation/reoxygenation; TA: tormentic acid; PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate.

to elucidate their underlying molecular mechanisms, with the goal of developing effective strategies to mitigate I/R-induced damage. The work herein resolves this gap via the evaluation of protective effect of tormentic acid (TA), a naturally occurring triterpenoid, in an *in vitro* I/R injury model. It is demonstrated that TA attenuates neuronal damage by suppressing microglia-mediated inflammation and inhibiting NF-κB signaling. Neuronal death during I/R injury is the result of not only energy failure but also the dysregulation of cellular homeostasis, characterized by suppressed cell viability, elevated apoptosis, and sustained inflammation [12]. The interplay between neuronal cells and activated microglia further aggravates the injury process. In particular, microglia being the CNS' resident immune cells are rapidly activated under ischemic conditions and release a broad spectrum of pro-inflammatory cytokines, which contribute to neuronal apoptosis and impair recovery [13]. In the work herein, it was observed that OGD/R treatments substantially decreased HT22 cell viability and induced apoptosis, as evidenced by decreased Bcl-2 and enhanced Bax expressions. Moreover, conditioned media from OGD/R-stimulated BV2 microglia further exacerbated neuronal apoptosis, highlighting the detrimental role of microglial-mediated inflammation in I/R injury. These findings underscore the importance of targeting both intrinsic neuronal responses and microglial-derived signals in developing neuroprotective interventions.

TA being a pentacyclic triterpenoid extracted from various medicinally important plants has attained focus because of broad pharmacological characteristics such as anti-apoptotic, antioxidant, and anti-inflammatory effects [14]. Previous studies have demonstrated its efficacy in models of neurodegeneration, arthritis, liver fibrosis, and cardiovascular disease [9, 15, 16]. In our model, TA pretreatment significantly restored neuronal viability under OGD/R conditions, reduced LDH release, suppressed apoptosis, and attenuated pro-inflammatory cytokines production in microglia. Notably, TA-treated microglial conditioned medium decreased neuronal apoptosis compared to OGD/R-activated microglial medium, suggesting that TA exerts indirect neuroprotective effects by modulating the microglial secretome. These results collectively support the notion that TA protects neurons during ischemia-reperfusion through non-cell-autonomous and cell-autonomous processes.

Multiple research works have reported advantageous impacts of TA across various disease contexts. For instance, TA inhibits IL-1β-induced apoptosis in chondrocytes and reduces

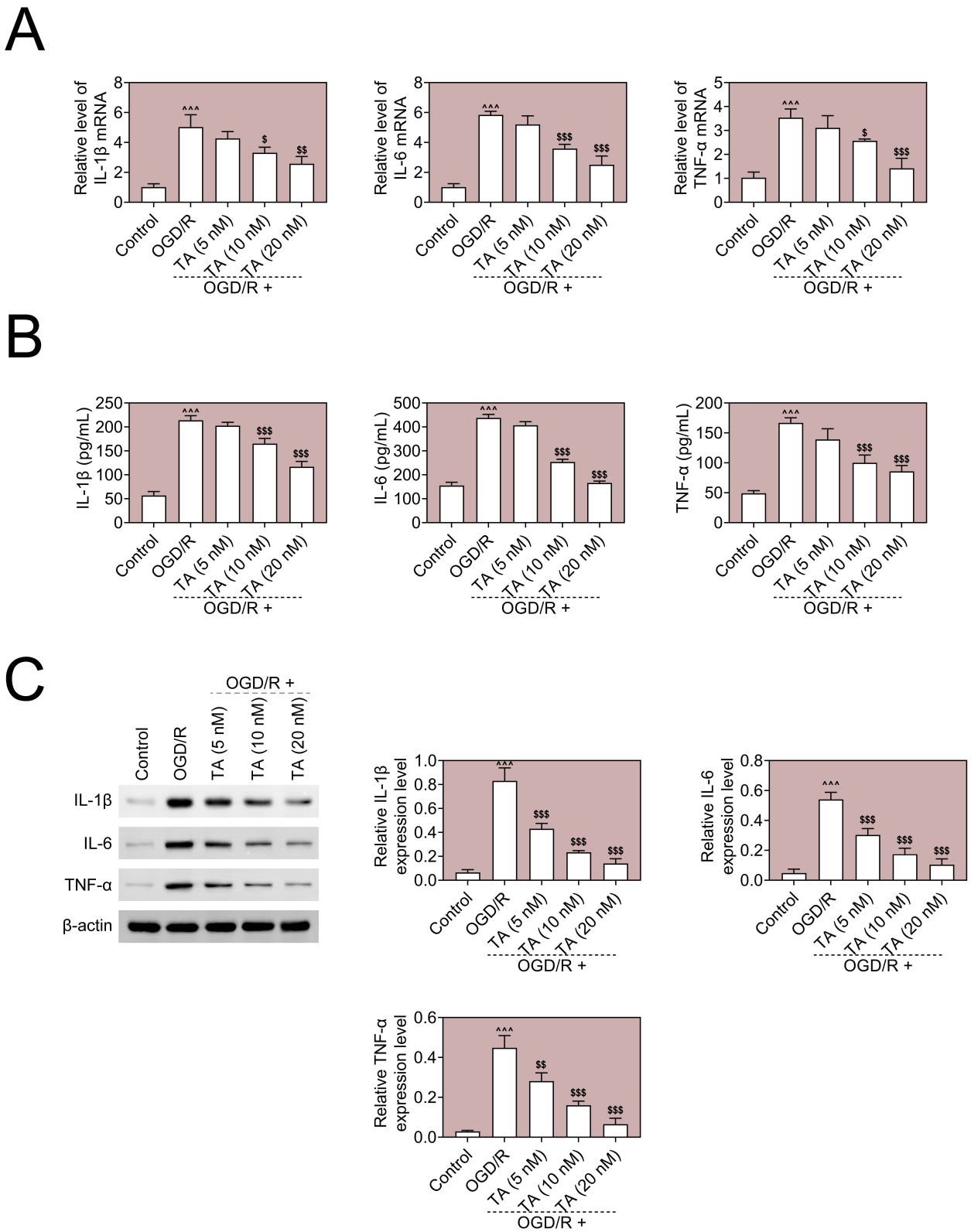

FIGURE 4. Tormentic acid suppresses inflammatory cytokine expression in BV2 microglial cells following OGD/R. (A) mRNA levels of IL-1β, IL-6, and TNF-α in BV2 cells after OGD/R with or without TA pretreatment (5, 10, 20 nM), as measured by quantitative real-time PCR. (B) Protein levels of IL-1β, IL-6, and TNF-α in culture supernatants, measured by Immunoblot. (C) Protein levels of IL-1β, IL-6, and TNF-α in culture supernatants, measured by ELISA. Data are presented as mean ± SD (n = 3). Each experiment was independently repeated three times $^{\wedge\wedge\wedge}p < 0.001$ *vs.* Control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs.* OGD/R group. OGD/R: oxygen-glucose deprivation/reoxygenation; TA: tormentic acid; IL: Interleukin; TNF-α: Tumor Necrosis Factor-alpha.

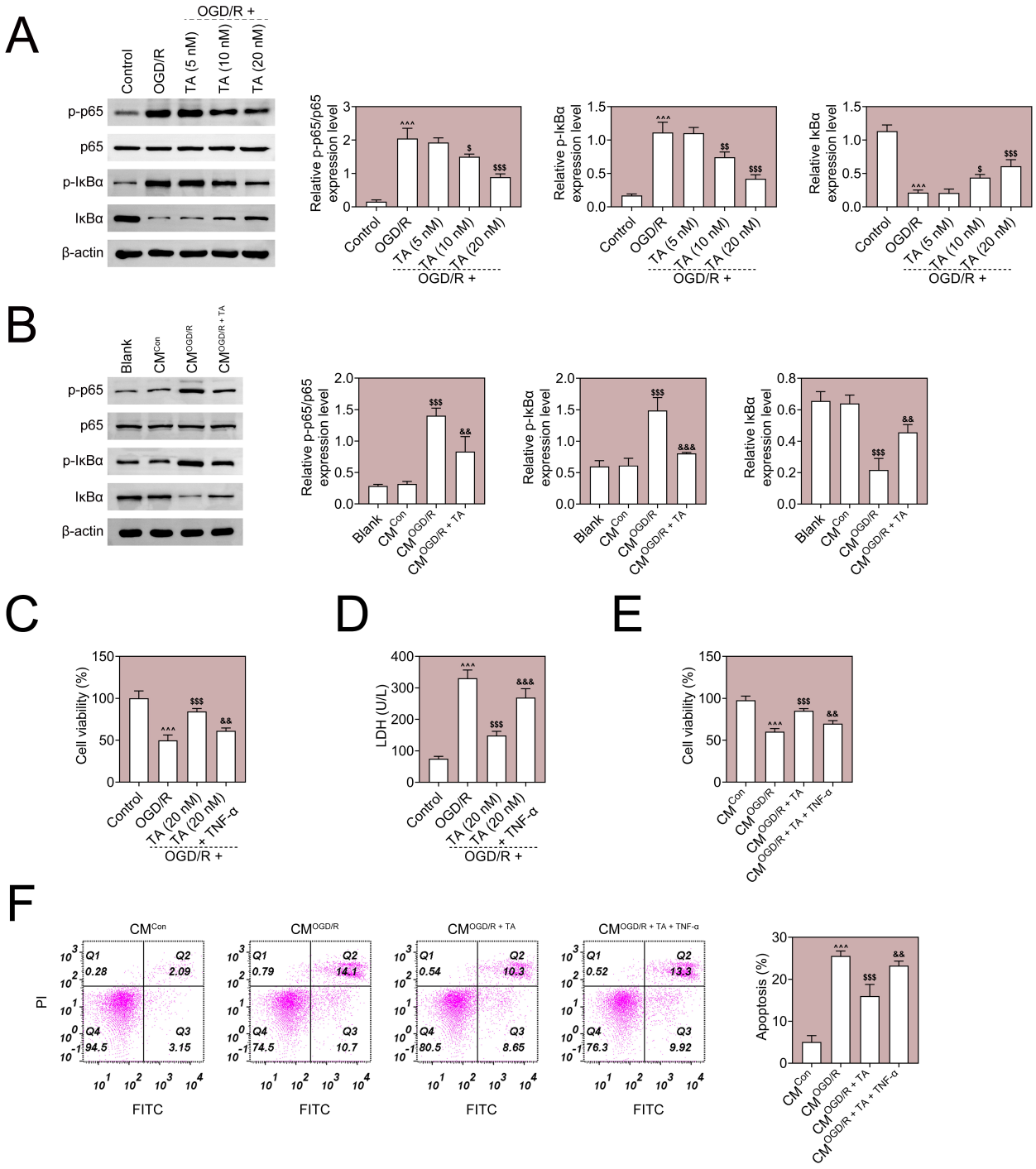

FIGURE 5. Tormentic acid inhibits NF-κB signaling activation in both BV2 and HT22 cells. (A) Western blot analysis of phosphorylated p65 (p-p65), total p65, phosphorylated IκBα (p-IκBα), and total IκBα in BV2 microglial cells exposed to OGD/R with or without TA pretreatment (5, 10, 20 nM). Quantification of protein levels relative to β-actin is shown. (B) Western blot analysis of NF-κB pathway proteins in HT22 cells cultured with conditioned media from different BV2 treatment groups. Protein quantification is shown on the right. Data are presented as mean $\pm$ SD (n = 3). (C) HT22 cells were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) with or without TA pretreatment (5, 10, 20 nM) or TNF-α, and cell viability was measured. (D) LDH release was evaluated to assess cell injury following OGD/R, TA, and/or TNF-α treatment. (E) HT22 cells were cultured with conditioned media (CM) from control BV2 cells, OGD/R-stimulated BV2 cells, or TA-pretreated OGD/R BV2 cells. Cell viability was assessed using the CCK8 assay. (F) Apoptosis of HT22 cells under different CM treatments was measured by Annexin V-FITC/PI staining and analyzed by flow cytometry. Representative dot plots and quantitative analysis of apoptotic rates are shown. Each experiment was independently repeated three times. $^{\wedge\wedge\wedge}p < 0.001$ *vs.* Control or Blank; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ *vs.* OGD/R or CM^{Con} group; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ *vs.* $CM^{OGD/R}$ group; $^{\&\&}p < 0.01$, $CM^{OGD/R}$ + TA + TNF-α *vs.* $CM^{OGD/R}$ + TA group. LDH: lactate dehydrogenase; PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate.

hepatic fibrosis. In Alzheimer models, TA ameliorates cognition deficiency and neuroinflammation, while in Parkinson's disorder, it alleviates oxidative stress [17]. Our findings extend these observations by demonstrating that TA also confers protection in an ischemic context. Unlike previous studies focusing solely on neuronal models, our approach incorporated both neuronal and microglial cell lines and assessed direct and indirect mechanisms, thereby providing more comprehensive understanding of TA's therapeutic potential against cerebral ischemia-reperfusion injury.

NF-κB axis is the central regulator of inflammation, apoptosis, and cell survival. It has key function regarding pathogenesis of ischemic brain injury [18]. NF-κB gets translocated towards nucleus upon activation and triggers multiple genes' transcription involved in inflammatory responses and programmed cell death [19]. In our study, OGD/R robustly activated NF-κB in both BV2 and HT22 cells, as indicated by increased phosphorylation of p65 and IκBα and degradation of IκBα. These molecular changes were significantly reversed by TA treatment, supporting the hypothesis that NF-κB inhibition underlies both anti-apoptotic and anti-inflammatory impacts of TA. Given NF-κB dual role in promoting inflammation and regulating cell fate, modulating this pathway provides a rational strategy for mitigating I/R injury.

In addition to inhibiting NF-κB, TA also interacts with other pathways like Wnt/β-catenin, extracellular regulated protein kinase 1/2 (ERK1/2), and phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt), depending on disease context. These pathways are interconnected with NF-κB and jointly modulate the cellular responses to stress and injury [20]. While our study focused on the NF-κB pathway, it is possible that TA exerts broader regulatory effects through crosstalk with these cascades. For example, activation of PI3K/Akt is known to promote neuronal survival, and its interplay with NF-κB may fine-tune the balance between pro- and anti-apoptotic signals. Future studies employing pathway-specific inhibitors or transcriptomic profiling will be necessary to delineate the full spectrum of TA's molecular targets in I/R injury.

Despite the promising findings, this study has several limitations. First, all experiments were conducted *in vitro* using immortalized cell lines, which may not fully recapitulate the complexity of *in vivo* ischemic brain injury. Second, we did not evaluate long-term functional outcomes or behavioral parameters, which are critical for assessing the translational potential of TA. Third, the specific receptors or upstream kinases through which TA modulates NF-κB signaling remain unclear. Further studies using primary neuronal and microglial cultures, animal stroke models, and in-depth mechanistic analysis are warranted to validate and extend our observations.

Our findings demonstrate TA's potent neuroprotection through NF-κB inhibition, but its multi-target nature warrants discussion of potential off-target effects and comparative advantages. Unlike synthetic NF-κB inhibitors (*e.g.*, BAY-11-7082) that cause broad immunosuppression, TA selectively attenuates pro-inflammatory microglial activation while preserving homeostatic functions is a critical distinction evidenced by our conditioned media experiments. This specificity may derive from TA's unique interactions with both canonical (p65/p50) and alternative (RelB/p52) NF-κB subunits, as reported for structurally similar triterpenoids [21, 22].

While the research herein gives valuable information regarding neuroprotective impacts of tormentic acid (TA) against I/R injury using HT22 and BV2 cell lines, it is important to acknowledge the inherent limitations of immortalized cell models. These systems may not completely replicate *in vivo* microenvironments' complexity, including cell-to-cell interactions, immune responses, and metabolic dynamics observed in primary neurons or animal models. To strengthen the translational relevance of our findings, further research must include primary neuronal and microglial culturing, which conducibly mimic physiological conditions, and validate the results in animal stroke models.

5. Conclusions

In summary, our study demonstrates that tormentic acid protects neuronal cells from I/R injury by enhancing cell viability, suppressing microglial-mediated inflammatory responses, blocking NF-κB signaling activation, and inhibiting apoptosis.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

CC—designed the study and carried them out. CC and YD—supervised the data collection; analyzed the data; interpreted the data; prepared the manuscript for publication and reviewed the draft of the manuscript. Both authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Ren K, Pei J, Guo Y, Jiao Y, Xing H, Xie Y, *et al*. Regulated necrosis pathways: a potential target for ischemic stroke. Burns & Trauma. 2023; 11: tkad016.

[2] Wei Z, Yu H, Zhao H, Wei M, Xing H, Pei J, *et al*. Broadening horizons: ferroptosis as a new target for traumatic brain injury. Burns & Trauma. 2024; 12: tkad051.

[3] Wang S, Shi X, Xiong T, Chen Q, Yang Y, Chen W, *et al*. Inhibiting mitochondrial damage for efficient treatment of cerebral ischemia-reperfusion injury through sequential targeting nanomedicine of neuronal mitochondria in affected brain tissue. Advanced Materials. 2024; 36: e2409529.

[4] Zhang M, Liu Q, Meng H, Duan H, Liu X, Wu J, *et al*. Ischemia-reperfusion injury: molecular mechanisms and therapeutic targets. Signal transduction and targeted therapy. 2024; 9: 12.

[5] Bai M, Cui N, Liao Y, Guo C, Li L, Yin Y, *et al*. Astrocytes and microglia-targeted Danshensu liposomes enhance the therapeutic effects on cerebral ischemia-reperfusion injury. Journal of Controlled Release. 2023; 364: 473–489.

[6] Zhang S, Chen Q, Jin M, Ren J, Sun X, Zhang Z, *et al*. Corrigendum to "Notoginsenoside R1 alleviates cerebral ischemia/reperfusion injury by inhibiting the TLR4/MyD88/NF-κB signaling pathway through microbiota-gut-brain axis" [Phytomedicine 128 (2024) 155530]. Phytomedicine. 2025; 141: 156635.

[7] Chen F, Wang W, Cai X, Yu H, Qu C, Zhang X, *et al*. Methyl jasmonate reduces the inflammation and apoptosis of HK-2 cells induced by LPS by regulating the NF-κB pathway. Signa Vitae. 2021; 17: 218–224.

[8] Heckmann M, Karlsberger L, Blank-Landeshammer B, Klanert G, Sadova N, Stadlbauer V, *et al*. 3-*O*-trans-*p*-coumaroyl esterification enhances the anti-inflammatory effects of tormentic acid by targeting NF-κB signaling. Redox Biology. 2025; 85: 103731.

[9] Lin X, Wei Y, Li Y, Xiong Y, Fang B, Li C, *et al*. Tormentic acid ameliorates hepatic fibrosis *in vivo* by inhibiting glycerophospholipids metabolism and PI3K/Akt/mTOR and NF-κB pathways: based on transcriptomics and metabolomics. Frontiers in Pharmacology. 2022; 13: 801982.

[10] Yue Y, Qiao B, Jiang X. Tormentic acid confers protection against oxidative stress injury in rats with Parkinson's disease by targeting the Wnt/β-catenin signaling pathway. Cellular and Molecular Biology. 2020; 66: 32–36.

[11] Liu D, Mei W, Zhou Y, Xi H. Targeting TRPM channels for cerebral ischemia–reperfusion injury. Trends in Pharmacological Sciences. 2024; 45: 862–867.

[12] Zhang H, Feng Y, Si Y, Lu C, Wang J, Wang S, *et al*. Shank3 ameliorates neuronal injury after cerebral ischemia/reperfusion via inhibiting oxidative stress and inflammation. Redox Biology. 2024; 69: 102983.

[13] Zhou H, Yan L, Huang H, Li X, Xia Q, Zheng L, *et al*. Tat-NTS peptide protects neurons against cerebral ischemia-reperfusion injury via ANXA1 SUMOylation in microglia. Theranostics. 2023; 13: 5561–5583.

[14] Yang AY, Kim JY, Gwon MG, Kwon HH, Leem J, Jeon EJ. Protective effects of tormentic acid on unilateral ureteral obstruction-induced renal injury, inflammation, and fibrosis: a comprehensive approach to reducing oxidative stress, apoptosis, and ferroptosis. Antioxidants. 2024; 14: 13.

[15] He JY, Li J, Zhang YY, He HB, He YM, Xu DX, *et al*. Tormentic acid, a triterpenoid isolated from the fruits of *Chaenomeles speciose*, protected indomethacin-induced gastric mucosal lesion via modulating miR-139 and the CXCR4/CXCL12/PLC/PKC/Rho a/MLC pathway. Pharmaceutical Biology. 2023; 61: 1343–1363.

[16] Kuo PJ, Chen LT, Li SM, Chen ZR, Chen JJ. Pharmacological and molecular docking investigation of leaves of *Eriobotrya japonica*: antioxidant, enzyme inhibition, and anti-inflammatory effects. Antioxidants. 2025; 14: 413.

[17] Tao Y, Bao J, Zhu F, Pan M, Liu Q, Wang P. Ethnopharmacology of Rubus idaeus Linnaeus: a critical review on ethnobotany, processing methods, phytochemicals, pharmacology and quality control. Journal of Ethnopharmacology. 2023; 302: 115870.

[18] Wang CP, Li JL, Zhang LZ, Zhang XC, Yu S, Liang XM, *et al*. Isoquercetin protects cortical neurons from oxygen-glucose deprivation-reperfusion induced injury via suppression of TLR4-NF-κB signal pathway. Neurochemistry International. 2013; 63: 741–749.

[19] Bai L, Li N, Li H, Dai Y, Bai Y, Ma F, *et al*. Macrophage-mimetic liposomes co-delivering ceria and Ac2-26 peptide for penumbra protection in ischemic stroke. Journal of Nanobiotechnology. 2025; 23: 488.

[20] Wang L, Zhu J, Meng M, Zhu S, Ma Y, Zhou T, *et al*. Inhibition of the MyD88/NF-κB pathway alters the Th1/Th2 balance to exacerbate liver injury and hepatic fibrosis in alveolar echinococcosis. FASEB Journal. 2025; 39: e70472.

[21] Zhang H, Meng R, Zhang F, Chen A, Ge H, Chen W, *et al*. IDO1 promotes *Echinococcus multilocularis* infection by regulating the formation of neutrophil extracellular traps. Veterinary Research. 2025; 56: 131.

[22] Sun J, Wang R, Chao T, Peng J, Wang C, Chen K, *et al*. Ginsenoside Re inhibits myocardial fibrosis by regulating miR-489/myd88/NF-κB pathway. Journal of Ginseng Research. 2023; 47: 218–227.

How to cite this article: Can Chen, Yue Dong. Tormentic acid attenuates neuronal injury caused by ischemia-reperfusion via suppression of microglial mediated neuroinflammation. Signa Vitae. 2025; 21(10): 107-116. doi: 10.22514/sv.2025.148.